(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 May 2003 (01.05.2003)

PCT

(10) International Publication Number
WO 03/035850 A1

(51) International Patent Classification[7]: C12N 5/00, A61L 15/40

(21) International Application Number: PCT/GB02/04829

(22) International Filing Date: 25 October 2002 (25.10.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

0125735.1	26 October 2001 (26.10.2001)	GB
0208930.8	12 April 2002 (12.04.2002)	GB
0208919.1	19 April 2002 (19.04.2002)	GB
0219798.6	27 August 2002 (27.08.2002)	GB

(71) Applicant *(for all designated States except US)*: **CELLTRAN LIMITED** [GB/GB]; Firth Court, Western Bank, Sheffield S10 2TN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HADDOW, David** [GB/GB]; Celltarn Limited, Firth Court, Western Bank, Sheffield S10 2TN (GB). **MACNEIL, Sheila** [GB/GB]; Celltran Limited, Firth Court, Western Bank, Sheffield S10 2TN (GB).

(74) Agent: **HARRISON GODDARD FOOTE**; 31 St Saviourgate, York Y01 8NQ (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/035850 A1

(54) Title: CELL TRANSFER SUBSTRATE

(57) Abstract: The invention relates to a substrate which has a coating which is obtainable by plasma polymerisation and provides a surface which cells can attach to, proliferate on and then detach from to invade and repair a wound, typically a cutaneous wound; and including cell culture conditions which minimise the likelihood of xenobiotic contamination of treated patients.

CELL TRANSFER SUBSTRATE

The invention relates to a substrate which has a coating which is obtainable by plasma polymerisation and provides a surface which cells can attach to, proliferate on and then detach from to invade and repair a wound, typically a cutaneous wound.

Tissue engineering is an emerging science which has implications with respect to many areas of clinical and cosmetic surgery. More particularly, tissue engineering relates to the replacement and/or restoration and/or repair of damaged and/or diseased tissues to return the tissue and/or organ to a functional state. For example, and not by way of limitation, tissue engineering is useful in the provision of skin grafts to repair wounds occurring as a consequence of: contusions, or burns, or failure of tissue to heal due to venous or diabetic ulcers.

Skin is a highly complex organ covering the external surface of the body. Skin functions, amongst other things, to prevent water loss from the body and to act as a protective barrier against the action of physical, chemical or infectious agents. Skin has an elastic property and varies in thickness from 0.5 mm, on for example the eyelids, to 4 mm on for example the palms and soles.

Skin is composed of two layers. The outer layer, which is comparatively thin is called the epidermis. It is several cells thick and has an external layer of dead cells that are constantly shed from the surface and replaced from below by a basal layer of cells, called the stratum germinativum. The epidermis is composed predominantly of keratinocytes which make up over 95% of the cell population, the rest include dendritic cells such as Langerhans and pigmented cells called melanocytes. It is essentially cellular and non vascular, there being relatively little extra cellular matrix except for the layer of collagen and other proteins beneath the basal layer of keratinocytes. Keratinocytes of the basal layer are constantly dividing, and daughter cells subsequently move outwards, during which they undergo a period of differentiation and are eventually sloughed off from the surface.

The favoured substratum for supporting the attachment and proliferation and growth of cells is collagen I. For example, keratinocytes seeded or deposited onto collagen-glycosaminoglycan (C-GAG) substrates and grafted to burns to form a cultured skin substitute (CSS), developed into permanent skin tissue after 14-28 days. Keratinocytes are also able to grow *in vitro* on synthetic hydrophilic polymer supports. Keratinocytes have been grafted onto poly(hydroxyethyl methacrylate) supports and these have shown improved wound bed healing, with no difference in the cytokeratin pattern of the unreconstructed epidermis and normal human skin.

Although collagen is the preferred substrate for culturing skin cells and transferring said cells to a wound bed, there are disadvantages in using this substrate.[1,2] For example, collagen has to be prepared, typically from a cadaver, which takes time and expense. Moreover, it is important to completely de-cellularise the collagen to reduce immune rejection and the likelihood of transferring infectious agents to the recipient of the donated collagen. Methods to sterilise collagen-containing tissues are known in the art but many result in a collagen base which lacks the consistency of native collagen. There is therefore a need to develop new substrates which support cell proliferation and transfer to a wound bed to promote wound repair.

A substrate suitable for this purpose would typically have the following characteristics: (i) be pliable; (i.e. typically a preferred thickness not greater than 0.2mm (e.g < 200 μm), and bi-axially flexible, making it suitable for conformability to a wound. (ii) preferably transparent; (iii) able to be sterilised (e.g.by γ-radiation),; and (iv) inexpensive to manufacture and easy to prepare; be in film form have a low Tg and be semi or non-crystalline. This is in addition to having the characteristics of being able to support cell attachment, growth and detachment of cells to promote wound healing. Examples of substrates having some or all of these characteristics are readily available.

We have identified substrates which have these characteristics when treated by plasma polymerisation, to provide a functionalised surface coating. Plasma polymerisation is a

technique which allows an ultra-thin (eg ca.200nm) cross linked polymeric film to be deposited on substrates of complex geometry and with controllable chemical functionality. As a consequence, the surface chemistry of materials can be modified, without affecting the bulk properties of the substrate so treated. Plasmas or ionised gases are commonly excited by means of an electric field. They are highly reactive chemical environments comprising ions, electrons, neutrals (radicals, metastables, ground and excited state species) and electromagnetic radiation. At reduced pressure, a regime may be achieved where the temperature of the electrons differs substantially from that of the ions and neutrals. Such plasmas are referred to as "cold" or "non-equilibrium" plasmas. In such an environment many volatile organic compounds (eg volatile alcohol containing compounds, volatile acid containing compounds, volatile amine containing compounds, or volatile hydrocarbons, neat or with other gases, eg Ar, have been shown to polymerise (H.K. Yasuda, Plasma Polymerisation, Academic Press, London 1985) coating both surfaces in contact with the plasma and those downstream of the discharge. The organic compound is often referred to as the "monomer". The deposit is often referred to as "plasma polymer". The advantages of such a mode of polymerisation potentially include: ultra-thin pin-hole free film deposition; plasma polymers can be deposited onto a wide range of substrates; the process is solvent free and the plasma polymer is free of contamination. Under conditions of low power, plasma polymer films can be prepared which retain a substantial degree of the chemistry of the original monomer. For example, plasma polymerised films of acrylic acid contain the carboxyl group (O'Toole L., Beck A.J., Short R. D., Macromolecules, 1996, 29, 5172-5177). The low power regime may be achieved either by lowering the continuous wave power, or by pulsing the power on and off (Fraser S., Barton D, Bradley J.W., Short R.D, J. Phys. Chem. B., 2002, 22 (106) 5596-5608).

Co-polymerisation of one or more compounds having functional groups with a hydrocarbon allows a degree of control over surface functional group concentrations in the resultant plasma copolymer (PCP) (Beck, A.J, Jones F.R., Short R.D., Polymer 1996, 37(24) 5537-5539). Suitably, the monomers are ethylenically

unsaturated. Thus the functional group compound maybe unsaturated carboxylic acid, alcohol or amine, for example, whilst the hydrocarbon is suitably an alkene.

The use of a low plasma power/monomer (W/F) flow rate ratio produces a plasma polymer/co-polymer in which the acid functionality of the acid-containing monomer (in this example, acrylic acid) is largely preserved intact (retained) from the plasma-gas to the plasma polymer/co-polymer deposit. These deposits do contain other functional groups (e.g. hydroxyls arising from post plasma oxidation) but are described in WO0078928 as "high acid retention", reflecting the high degree of acid retention from the plasma gas into the plasma polymer film.

References herein to include surfaces which have amounts of 2-20% surface acid, or 5-20% surface acid, and in excess of 20% surface acid. The percentages refer to the percent of carbon atoms in this type of environment. For example, 20 % acid means that 20 of every one hundred carbons in the plasma polymer is in an acid-type environment.

By plasma polymerisation, it is also possible to deposit a range of other types of surfaces. For example the plasma polymerisation of ethylene oxide-type molecules (eg. tetraethyleneglycol monoallyl ether) to form 'non-fouling' surfaces is well known (Y. J. Wu et al., Colloids and Surfaces B: BioInterfaces 18 (2000) 235-248). It is also possible to deposit perfluoro-compounds (i.e. perfluorohexane, hexafluoropropylene oxide) to form hydrophobic/superhydrophobic surfaces (ref.).

Overall the plasma polymerisation is advantageous because the surfaces thus deposited have unique chemical and physical characteristics.

The present application relates to the provision of a structure, comprising a substrate treated with a plasma polymerised coating to provide a surface to which cells adhere, proliferate and detach.

Examples of substrates include, olefins which includes polyethylene [PE] (very low density, linear low density, chlorinated (<20%) and aliphatic polyolefins (other than polyethylene)) eg polypropylene (PP) polybut-1-ene (atactic), polyisobutylene and diene rubbers (eg natural rubber, styrene butadiene rubber (incl. latex) butyl rubber, nitrile rubber, polybutadiene, polyisoprene ethylene-propylene rubber, and polychlorprene). PE and other aliphatic olefins may contain additives such as -antioxidants, antiozonates, softners, processing aids, blowing agents, pigments, and filers.

Further examples include, ethylene co-polymers such as ethylene vinyl actetate and ethylene ethyl acrylate; vinyl chloride polymers such as polyvinyl chloride, polyvinyl acetate, polyvinyl alcohol which can include stabilisers, plasticizers, lubricants, fillers and miscellaneous additives; acrylics such as acrylic rubbers and acrylic polymer blends; styrene based plastics such as styrene isoprene and styrene thermoplastic elastomers; polyamides such as polyamide 12 and polyamide co-polymers; silicones polymers, an example of which is polydimethyl siloxane, and silicone rubbers; polyurethanes; polyurethane rubbers; and polysulphides.

According to an aspect of the invention there is provided a cell transfer substrate comprising at least one surface wherein said surface is obtainable by plasma polymerisaton of a monomer preparation to provide a plasma coated surface.

According to a further aspect of the invention there is provided a cell transfer substrate comprising at least one surface which is functionalised and wherein said surface is obtainable by plasma polymerisaton of a monomer preparation to provide a plasma coated surface.

In a preferred embodiment of the invention the surface contains either acid, alcohol or amine functionalities.

In a preferred embodiment of the invention and said surface is at least 2% acid.

In a further preferred embodiment of the invention said surface is at least 5% acid.

In a preferred method of the invention said cell culture surface comprises a plasma polymer comprising an acid content of at least 2%. Preferably said acid content is 2-20% or 5-20%. Alternatively, said acid content is greater than 20%. The percentages refer to the percent of carbon atoms in this type of environment. The acid content of the surface is determined by methods herein disclosed and are known in the art. For example, percent acid maybe measured by x-ray photoelectron spectroscopy (XPS).

In an alternative preferred embodiment of the invention said surface has been treated by plasma polymerisation with a volatile acid.

In a preferred embodiment of the invention said surface has been treated by plasma polymerisation with a volatile alcohol.

In a further alternative preferred embodiment of the invention said surface has been treated by plasma polymerisation with a volatile amine.

In a still further preferred embodiment of the invention said surface has been treated by plasma polymerisation with a mixture of volatile acid and volatile hydrocarbon.

Polymerisable monomers that may be used in the practice of the invention preferably comprise unsaturated organic compounds such as, olefinic carboxylic acids and carboxylates, olefinic amines, olefic alcohols, olefinic nitrile compounds, oxygenated olefins, halogenated olefins and olefinic hydrocarbons. Such olefins include vinylic and allylic forms. The monomer need not be olefinic, however, to be polymerisable. Cyclic compounds such as cyclohexane, cyclopentane and cyclopropane are commonly polymerisable in gas plasmas by glow discharge methods. Derivatives of these cyclic compounds, such as 1, 2- diaminocyclohexane for instance, are also commonly polymerisable in gas plasmas. Particularly preferred are polymerisable monomers containing hydroxyl, amino or carboxylic acid groups. Of these, particularly

advantageous results have been obtained through use of acrylic acid or allyl amine. Mixtures of polymerisable monomers may be used. Additionally, polymerisable monomers may be blended with other gases not generally considered as polymerisable in themselves, examples being argon, nitrogen and hydrogen. The polymerisable monomers are preferably introduced into the vacuum chamber in the form of a vapour. Polymerisable monomers having vapour pressures less than 1.3×10^{-2}mbar (1.3 Pa) are not generally suitable for use in the practice of this invention.

Polymerisable monomers having vapour pressures of at least 6.6×10^{-2}mbar (6.6 Pa) at ambient room temperature are preferred. Where monomer grafting to plasma pressures of at least 1.3 mbar (130Pa) at ambient conditions are particularly preferred polymerisate deposits is employed, polymerisable monomers having vapour.

To maintain desired pressure levels, especially since monomer is being consumed in the plasma polymerisations operation, continuous inflow of monomer vapour to the plasma zone is normally practiced. When non polymerisable gases are blended with the monomer vapour, continuous removal of excess gases is accomplished by simultaneously pumping through the vacuum port to a vacuum source, indeed this is the case when employing polymerisable monomers. Since some non-polymerisable gases are often evolved from glow discharge gas plasmas, it is advantageous to control gas plasma pressure at least in part through simultaneous vacuum pumping during plasma polymerisate deposition on a substrate in the process of this invention.

Monomers suited for this invention include, fully saturated and unsaturated carboxylic acid compounds up to 20 carbon atoms. More typically 2-8 carbons. Ethylenically unsaturated compounds (especially α,β unsaturated carboxylic acids) including acrylic acid, methacrylic acid. Saturated including ethanoic acid and propanoic acid. Alterntaively, compounds that can be plasma polymerised that readily hydrolyse to give carboxylic acid functionalities, e.g. organic anhydrides (e.g. maleic anhydride) acyl chlorides may be used.

In a further preferred method of the invention said polymer comprises an acrylic acid monomer with at least 2% or 5% acid content. Preferably said acid content is between 2% and 20% or between 5% and 20%. Alternatively, the acid content can be greater than 20%.

In a further preferred method of the invention said polymer comprises an acid co-polymer. The copolymer is prepared by the plasma polymerisation of an organic carboxylic acid (or anhydride) with a saturated (alkane) or unsaturated (alkene, diene or alkyne) hydrocarbon. The hydrocarbon would be of up to 20 carbons (but more usually of 4- 8). Examples of alkanes are butane, pentane and hexane. Examples of alkenes are butene and pentene. An example of a diene is 1-7 octadiene-. The co-monomer may also be aromatic-containing e.g. styrene.

Co-plasma polymerisation may be carried out using any ratio of acid : hydrocarbon, but will be typically using an acid: hydrocarbon ratio between the limits of 100(acid):0(hydrocarbon) to 20 (acid):80 (hydrocarbon) and any ratio between these limits.

Plasma polymerised amines are also within the scope of the invention, for example, fully saturated primary, secondary or tertiary amines (e.g. butyl amine, propyl amine, heptylamine) or unsaturated e,g, allyl amine, which would be up to 20 carbons but more typically 4-8 carbons. Amines could be co-polymerised with hydrocarbons as above.

Plasma polymerised alcohols are also within the scope of this invention. Alcohols could be co-polymerised with hydrocarbons.

The glow discharge through the gas or blend of gases in the vacuum chamber may be initiated by means of an audiofrequency, a microwave frequency or a radiofrequency field transmitted to or through a zone in the vacuum chamber. Particularly preferred is the use of a radiofrequency (RF) discharge, transmitted through a spatial zone in the vacuum chamber by an electrode connected to an RF signal generator. A rather broad

range of RF signal frequencies starting as low as 50 kHz may be used in causing and maintaining a glow discharge through the monomer vapor. In commercial scale usage of RF plasma polymerisation, an assigned radiofrequency of 13.56 MHz may be more preferable to use to avoid potential radio interference problems as with examples given later. Typically, using the composite ratio of W/FM, as described by Yasuda (1985) the power loading should be $< 10^9$ J/kg to achieve functional group retention in plasma polymers. (W= power (J/min); F= flow rate (mole/min); M = average molecular mass (kg/mol) [Although flow rate is given as sccm, this is in fact not strictly correct. Conversion from sccm to mol/min can be readily performed by dividing scccm by 22, 400).

The glow discharge need not be continuous, but may be intermittent in nature during plasma polymerisate deposition. Or, a continuous glow discharge may be employed, but exposure of a substrate surface to the gas plasma may be intermittent during the overall polymerisate deposition process. Or, both a continuous glow discharge and a continuous exposure of a substrate surface to the resulting gas plasma for a desired overall deposition time may be employed. The plasma polymerisate that deposits onto the substrate generally will not have the same elemental composition as the incoming polymerisable monomer (or monomers). During the plasma polymerisation, some fragmentation and loss of specific elements or elemental groups naturally occurs. Thus, in the plasma polymerisation of acrylic acid, carboxyl content of the plasma polymerisate is typically lower than would correspond to pure polyacrylic acid. Similarly, in the plasma polymerisation of allylamine, nitrogen content of the plasma polymerisate is typically lower than would correspond to pure polyallylamine. Exposure time to either of these unreacted monomers in the absence of a gas plasma, as through intermittent exposure to a glow discharge, allows for grafting of the monomer to the plasma polymerisate, thereby increasing somewhat the level of the functional group (carboxylic acid or amine) in the final deposit. Time intervals between plasma exposure and grafting exposure can be varied from a fraction of a second to several minutes.

In still a further preferred embodiment of the invention said cell transfer substrate is provided by coating a substrate with a plasma copolymer of an acidic monomer. For example and not by limitation, acrylic acid and a hydrocarbon, for example 1,7 octadiene. Ideally said acrylic acid provides 50-100% and 1,7 octadiene 50-0% in the gas feed.

In a preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: the olefins, which includes polyethylene (very low density, linear low density, chlorinated (<20%) and aliphatic polyolefins (other than polyethylene) eg polypropylene (PP) polybut-1-ene (atactic) and polyisobutylene).

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: diene rubbers (eg natural rubber, styrene butadiene rubber (incl. latex) butyl rubber, nitrile rubber, polybutadiene, polyisoprene ethylene-propylene rubber, polychlorprene.

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: ethylene co-polymers such as ethylene vinyl actetate and ethylene ethyl acrylate.

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: vinyls such as vinyl chloride polymers e.g. polyvinyl chloride, polyvinyl acetate, polyvinyl alcohol.

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: acrylics such as acrylic rubbers and acrylic polymer blends.

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: styrene based plastics such as styrene isoprene and styrene thermoplastic elastomers.

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: polyamides such as polyamide 12 and polyamide co-polymers.

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of: silicones polymers an example of which is polydimethyl siloxane and silicone rubbers; polyurethanes; polyurethane rubbers; and polysulphides.

A preferred example of a substrate is one comprising a vinyl polymer. A vinyl polymer is a polymer based upon repeat units having the formula (CH_2CHX), e.g. polyvinylchloride $(CH_2CHCl)_n$

Surprisingly, vinyl polymers, for example polyvinyl chloride (PVC), treated by plasma polymerisation to provide acid surfaces, serve as excellent substrates for the attachment and growth of keratinocytes. Keratinocytes represent a particular challenge for these studies because they will undergo irreversible terminal differentiation on many substrates. The irreversible differentiation of these cells results in loss of the capacity to migrate or form colonies, properties which are required in considering transfer of keratinocytes from supporting surfaces to wound beds to achieve re-epithelialization. Moreover, in clinical studies, wounds which are treated with vinyl polymers (coated by plasma polymerisation) delivering human keratinocytes perform as well as collagen, hitherto a preferred substrate for delivering cells for wound healing.

In a preferred embodiment of the invention said acid surface is 2-20% or 5-20%. Preferably said acid surface is greater than 20%.

In a preferred embodiment of the invention said surface has been treated by plasma polymerisation with a volatile acid. For example a carboxylic acid (especially α,β -

unsaturated carboxylic acid, for example acrylic, or methacrylic acid) or alternatively, propanoic acid, or maleic anhydride). Ideally said acid is provided by acrylic acid.

More preferably still said plasma polymer is a co-polymer. Ideally said co-polymer comprises at least one organic acid monomer with at least one hydrocarbon. Ideally said hydrocarbon is an alkene, eg a diene such as, for example, octa-1,7-diene. However, a saturated alkane, e.g. hexane may be used.

In still a further preferred embodiment of the invention, said surface is provided by plasma coating said vinyl polymer with a co-polymer of an ethylenically unsaturated acidic monomer. For example and not by way of limitation, acrylic acid and an (ethylenically) unsaturated hydrocarbon, for example, 1,7-octadiene. Ideally the plasma polymerisation is performed using a gas feed comprising 50-100% acrylic acid and 0-50% 1,7-octadiene (by volume in the gas flow).

In a further preferred embodiment of the invention said substrate comprises a polymer selected from the group consisting of polyvinyl chloride, polypropylene, silicone and polyhydroxybutyrate. Examples include, PVC (PL 1240, Baxter), PVC (PL 146, Baxter), PVC (410 CU, Pall Medical), PVC (5550 Seta, Solmed), PVC (3226 Seta, Solmed), Polypropylene (7210, Solmed), Polyhydroxybutyrate (Goodfellow) and Silicone-poly dimethylsiloxane (Baxter).

In a further preferred embodiment of the invention said substrate comprises polyvinyl chloride.

According to a further aspect of the invention there is provided a substrate comprising a plasma coated polymer according to the invention and at least one mammalian cell. Preferably said substrate comprises a vinyl polymer.

In a preferred embodiment of the invention said mammalian cell is human.

In a further preferred embodiment of the invention said mammalian cell is selected from the group consisting of: epidermal keratinocytes; dermal fibroblasts; adult skin stem cells; embryonic stem cells; melanocytes, corneal fibroblasts, corneal epithelial cells, corneal stem cells; intestinal mucosa fibroblasts, intestinal mucosa keratinocytes,oral mucosa fibroblasts,oral mucosa keratinocytes, urethral fibroblasts and epithelial cells, bladder fibroblasts and epithelial cells, neuronal glial cells and neural cells, hepatocyte stellate cells and epithelial cells.

Preferably said cell is a skin cell. More preferably still said cell is a skin cell selected from the following group: keratinocyte; fibroblast; adult skin stem cell; embryonic stem cell; or melanocytes.

According to a yet further aspect of the invention there is provided a method to culture mammalian cells on a substrate comprising the steps of:

i) providing a preparation comprising;
   a) mammalian cells;
   b) a substrate according to the invention;
   c) cell culture medium sufficient to support the growth of said mammalian; and
ii) providing cell culture conditions which promote the proliferation of said mammalian cells on said substrate.

In a preferred method of the invention said substrate has attached thereto, fibroblast feeder cells.

In a further preferred method of the invention cells wherein said medium does not include serum.

In a preferred method of the invention said mammalian cells are human.

In a further preferred method of the invention said mammalian cells are selected from the group consisting of: epidermal keratinocytes; dermal fibroblasts; adult skin stem cells; embryonic stem cells; melanocytes, corneal fibroblasts, corneal epithelial cells, corneal stem cells; intestinal mucosa fibroblasts, intestinal mucosa keratinocytes,oral mucosa fibroblasts,oral mucosa keratinocytes, urethral fibroblasts and epithelial cells, bladder fibroblasts and epithelial cells, neuronal glial cells and neural cells, hepatocyte stellate cells and epithelial cells.

Preferabaly said mammalian cells are autologous, preferably autologous keratinocytes.

In a further preferred method of the invention said fibroblast feeder cells are human.

In a further preferred method of the invention said fibroblast feeder cells are human dermal fibroblasts or human oral fibroblasts. Preferably said feeder cells are autologous.

The direct culturing of mammalian cells on a substrate according to the invention under conditions herein disclosed has obvious benefits in tissue engineering since the fabrication of said substrate allows the culturing, implantation and transfer of cells to a wound to be repaired. The absence of serum and the use of autologous cells also minimizes the transfer of xenobiotic agents (e.g. viral agents, prions) from serum and/or feeder cells used in the culture of mammalian cells.

According to a further aspect of the invention there is provided a method to treat a mammal, preferably a human, suffering from a wound comprising contacting the wound with a mammalian cell-bearing substrate of the invention.

In a preferred method of the invention said wound is a cutaneous wound and said cell is a skin cell (e.g. keratinocyte; skin stem cell).

According to a further aspect of the invention there is provided a method to coat a substrate according to the invention comprising the steps of:

i) providing at least one organic monomer;

ii) creating a plasma of said organic monomer; and

iii) coating said substrate with said plasma.

According to a further aspect of the invention there is provided a method of preparing a substrate according to the invention comprising:

i) mixing a selected ratio of an ethylenically-unsaturated carboxylic acid monomer and an unsaturated hydrocarbon to form a gas feed;

ii) creating a plasma of said mixture; and

iii) coating a substrate with said plasma to provide a surface polymer/copolymer containing acid functionality.

i) coating said surface with said plasma.

In a preferred method of the invention said monomer is an acid monomer source comprising 30-99% acid monomer. Preferably said acid monomer source consists of a 100% acid monomer source. Preferably said method consists of a 100% acrylic acid source.

According to a further aspect of the invention there is provided a method to treat the substrate comprising the steps of:

i) providing a selected ratio of a monomer and a hydrocarbon in a gas feed;

ii) creating a plasma of said mixture;

iii) bringing into contact the substrate with said plasma mixture to provide a surface comprising a co-polymer.

In a preferred method of the invention said monomer is an acid monomer and said co-polymer is an acid co-polymer.

In a preferred method of the invention said plasma is created by means of electrical power input (radio frequency 13.56MHz), coupled by means of a copper coil or bands. The reactor volume would be typically in the range 2- 10 L (but may be larger or smaller) and the reactor is pumped by means of a double stage rotary pump to a base pressure approaching 10^{-4} mbar. In the case of replacing the rotary pump with a turbomolecular pump better base pressures can be achieved. The monomer pressure is in the range 10^{-1} mbar to 10^{-3} mbar and the monomer flow rate is 1-20 cm^3/ min (sccm). The power would be typically 0.1 –50W continuous wave. Those skilled in the art may adjust these parameters to produce like plasmas by pulsing on the micro or milli second time scales.

In a preferred method of the invention said acid is acrylic acid and said hydrocarbon is a diene and especially a di-unsaturated alkene, for example 1,7-octadiene.

In a further preferred method of the invention said plasma comprises 50-100% unsaturated acid, for example, acrylic acid and 0-50% hexane or diene, (for example, 1,7-octadiene) in the gas feed.

In yet a further preferred embodiment of the invention said plasma comprises the following ratios of acid (eg acrylic acid) and hexane or diene(eg1,7-octadiene);

Acid (eg Acrylic acid)	%	alkene (eg 1,7-octadiene %)
50		50
60		40
70		30
80		20
90		10
100		0

in the gas feed.

In a preferred method of the invention said substrate comprises a vinyl polymer.

It will be apparent to one skilled in the art that the creation of a plasma can use either low or high power and either a continuous wave or pulsed plasma.

Preferably said plasma power is created using a plasma power of 0 –50W and a flow rate of 0 – 20 sccm, usually under continuous wave conditions. However, in the instance of where a pulsed wave is used corresponding corrections are made to the plasma power and flow rate as is known by those skilled in the art.

In a preferred method of the invention said acid is acrylic acid and said hydrocarbon is a diene and especially a di-unsaturated alkene, for example 1,7-octadiene.

In a further preferred method of the invention said plasma comprises 50-100% unsaturated acid, for example, acrylic acid and 0-50% hexane or diene, (for example, 1,7-octadiene) in the gas feed.

In yet a further preferred embodiment of the invention said plasma comprises the following ratios of acid (eg acrylic acid) and hexane or diene(eg1,7-octadiene);

Acid		alkene
(eg Acrylic acid)	%	(eg 1,7-octadiene %)
50		50
60		40
70		30
80		20
90		10
100		0

in the gas feed.

An embodiment of the invention will now described, by example only and with reference to the tables and figures wherein

Table 1 illustrates medical grade carrier polymers;

Table 2 summarises the results of X-ray photoelectron spectroscopy of plasma polymerised surfaces. A β-shifted carbon bonded to carboxylate ((C-COOH/R) at +0.7 eV from the hydrocarbon) of equal magnitude to the carboxylate has been added to the peak fit;

Table 3 illustrates a comparison of C 1s core line peak fit for as-treated and ethylene oxide sterilized PPs comprising 100% acrylic acid deposited on PVC (Solmed Vinylfoil 5550 Seta Glad);

Table 4 illustrates a comparison of cell attachment and transfer from various carrier films polymerised with 100% acrylic acid. Assessment of attachment is related to attachment of keratinocytes to collagen 1 coated PVC used as a positive control throughout for these experiments. Thus, +++ = good attachment equivalent to that of cells on collagen 1; ++ = reasonable attachment; + = poor attachment; 0 = no evidence of attachment. All results are based on polymers post ethylene oxide sterilisation (which was not found to significantly affect performance of the polymer for cell attachment). These results are based on two experiments with keratinocytes from different donors and in each experiment polymers were studied in duplicate;

Figure 1 illustrates C 1s peak fit for 100% Acrylic Acid PP on PVC carrier film (Solmed Vinylfoil, 5550 Seta);

Figure 2 illustrates MTT-ESTA assay showing keratinocyte attachment to surfaces after 24 hours;

Figure 3 illustrates MTT-ESTA assay showing keratinocytes remaining on surfaces after separation from DED;

Figure 4 illustrates MTT-ESTA assay showing the keratinocyte transfer from surfaces to DED after 4 days;

Figure 5a-5c illustrates the appearance of the DED post-staining for metabolically active cells where it can be seen that cells initially cultured on collagen I (Fig. 5a) showed the greatest transfer to the DED, cells cultured on a hydrocarbon PP surface showed little transfer (Fig. 5b) and cells cultured on the acid surfaces (Fig. 5c) showed a good level of transfer - in some cases, approximating 80% of that seen for cells coated on collagen I. This experiment was repeated in triplicate with similar results;

Figure 6a-6c illustrates the appearance of the epidermal layer formed after 4 days post-transfer of cells from (a) Collagen I, (b) 100% acrylic acid PP and (c) 50% acrylic acid PP surfaces. H&E staining showed that keratinocytes transferred from a Collagen I surface were closely attached to the basement membrane of the DED (Fig. 6a);

Figure 7a-i illustrates case studies of three patients undergoing tissue repair of toe ulcers which resulted from diabetes and caused by neuropathy; and

Figure 8 summarises a comparison of TranCell^{tm} with other transfer substrates characterised by how effectively keratinocytes invade wound bed substrates.

MATERIALS and METHODS

Plasma Polymerisation and Co-Polymerization

Acrylic acid and 1,7-octadiene were obtained from Aldrich Chemical Co. (UK). All monomers were used as received, after several freeze-pump/thaw cycles.

The monomer was aliquoted into 5ml batches and stored in a refrigerator until required for use. For each polymerisation one 5ml aliquot was used and then discarded. Prior to polymerisation the monomer was degassed using several freeze-pump/thaw cycles.

Polymerisations were carried out in cylindrical reactor vessels (of 8cm diameter and 50 cm length), evacuated by a two stage rotary pump. Stainless steel flanges were sealed to the glass vessel using viton 'o' rings. Plasma was sustained by a radio-frequency (13.56 MHz) signal generator and amplifier inductively coupled to the reactor vessel by means of an external copper coil. The base pressure in the reactor prior to polymerisation was always $< 1 \times 10^{-3}$ mbar.

Acrylic acid was polymerised using continuous wave plasma powers in the range of 1-10W and a total flow rates in the range 1-20sccm. Plasma polymers were deposited onto the polymer films, and clean silica glass cover slips or Al foil for XPS analysis. The pressure with the monomer flowing was typically 4.0×10^{-2} mbar.

Co-polymerisation using acrylic acid and 1,7-octadiene was carried out using the same range of power, flow rate and pressure conditions.

For all polymerisations and co-polymerisations a deposition time of typically 15 minutes was used. The monomers were allowed to flow for typically a further 20 minutes after the plasma was extinguished in order to minimise the up-take of atmospheric oxygen by the deposits on exposure to the laboratory atmosphere.

In order that the reactor vessels (and steel sample tray) were in a clean condition for each polymerisation, an etch with an oxygen plasma was carried out after each deposition. The oxygen gas was allowed to flow through the reactor at a pressure of 1.0×10^{-1} mbar. The plasma power was 50W and the etch time was one hour. XPS of a silica glass cover slip that had previously been polymerised with acrylic acid was examined by XPS to confirm that all the acid had been etched away.

<u>Plasma Polymerisation of Amine and Alcohol Monomers</u>

Following the procedure described above for acrylic acid, the same reactor design and plasma conditions (namely powers, pressures and flow rates), plasma polymers

containing amine or alcohol functionality were prepared from allyl amine and allyl alcohol monomers, respectively (Aldrich Chemical Co, UK). Plasma polymers were deposited onto the polymer films and clean silica glass coverslips or Al foil for XPS.

Copolymerisations using allyl amine or allyl alcohol and 1,7-octadiene were carried out in the same manner.

X-ray Photoelectron Spectroscopy

XP spectra were obtained on a VG CLAM 2 photoelectron spectrometer employing Mg K_α X-rays. Survey scan spectra (0-1100 eV) and narrow spectra were acquired for each sample using analyser pass energies of 50 and 20 eV respectively. Spectra were acquired using Spectra 6.0 software (R. Unwin Software, Cheshire, UK). Subsequent processing was carried out with Scienta data processing software (Scienta Instruments, Uppsala, Sweden). The spectrometer was calibrated using the Au 4f 7/2 peak position at 84.00 eV, and the separation between the C 1s and F 1s peak positions in a sample of PTFE measured at 397.2 eV, which compares well with the value of 397.19 eV reported by Beamson and Briggs[3].

Cell Culture and Transfer Experiment

Normal human adult keratinocytes (obtained from breast reductions and abdominoplasties) were isolated from the dermal/epidermal junction as previously described[4]. Cells were cultured in complete Green's media (Hams F10) which included cholera toxin (0.1 nM), hydrocortisone (0.4 μg/ml), adenine (1.8×10^{-4} M), triiodo-1-thyronine (2×10^{-7} M), insulin (5 mg/ml), transferring (5 μg/ml), glutamine (2×10^{-3} M), fungizone (0.625 μg/ml), penicillin (1000 IU/ml), streptomycin (1000 μg/ml) and 10% fetal calf serum. Cells were cultured at 37°C, in a 5% CO_2 atmosphere.

Freshly isolated cells were used for cell culture experiments. Collagen coated carrier polymer samples were prepared by air drying a solution of collagen I (32 $\mu g/cm^2$) in a 0.1 M acetic acid (200 μg/ml) in a laminar flow cabinet overnight.

Cells were seeded at densities of 12.0 x 10^6 cells/ml onto triplicate of the surfaces using a 10 mm internal diameter stainless steel ring to keep the samples flat within a 6 well (3.5 cm diameter) tissue culture plate. After 24 hours in culture, the keratinocyte attachment on one sample from each triplicate was determined using an MTT-ESTA assay. This gives an estimate of viable cell number, the assay having previously been shown to parallel increases in cell number for human keratinocytes[5]. Cells were incubated with 0.5 mg ml^{-1} of MTT in PBS for 40 minutes. The stain was then eluted with acidified isopropyl alcohol. The optical density at 540 nm with a protein reference wavelength of 630 nm was then determined.

DED was prepared from a split thickness skin graft (STSG) from abdominoplasties and breast reductions. The method is described in detail elsewhere[6], but briefly, STSG were glycerol pretreated prior to ethylene oxide sterilization, using a standard Sterivit™ process using a mixture of 15% ethylene oxide and 85% carbon dioxide at a pressure of 5.5 atmospheres at 55°C to ensure a concentration of 1200mg ethylene oxide per litre in a sterilization cycle of 30 minutes. Sterilized skin was hydrated in a large volume of sterile phosphate buffered saline (PBS) for 24 hours at 37°C. Skin was then immersed in 1M NaCl solution for 6-8 hours at 37°C and the epidermis removed using sterile forceps. Skin was then rehydrated in an excess of keratinocyte culture medium (KCM) for 48 hours at 37°C prior to use.

The remaining two samples from each triplicate were placed in contact with DED and Green's media added so that the surface/DED junction sat at the air/liquid interface. The DED/surface wound bed models were placed in an incubator at 37°C for 4 days, after which the surfaces were separated from the DED and the level of cell transfer from surface to DED assessed using the MTT assay, as described above. MTT of the DED required that the DED was incubated with MTT for 120 minutes before elution of the stain. After 4 days, a small sample of the DED was sent for hematoxylin-eosin (H&E) staining and histological evaluation. These samples were assessed for overall morphology, keratinocyte attachment to the dermis and extent of cell coverage.

The procedure detailed above was repeated for selected PPs deposited onto the eight medical grade polmers described in Table 1. All of these materials were ethylene oxide sterilized as outlined above, prior to the cell attachment and transfer experiments.

Co- Culture of keratinocytes plus fibroblasts in the presence and absence of serum

In addition to cuturing keratinocytes on their own on the PP surface we also developed co-culture of keratinocytes with human dermal fibroblasts under both serum containing and serum-free conditions.

Fibroblasts were obtained from the dermal layer of the skin after trypsinisation of a split-thickness skin graft, which was taken from specimens following routine surgery procedures (breast reduction and abdominoplasty), following washing in PBS and then minced finely with a scalpel and placed in 0.5% collagenase. Following centrifugation of the collagenase digest and elimination of the supernatant, the cells were resuspended in 10mls of fibroblast culture medium (FCM) in a T25 Flask. The flask is maintained at 37°C in a 5% CO_2 atmosphere.

Every 500ml of FCM consists of 438.75mls of Dulbecco's Modified Eagle's medium (DMEM), 50 mls of Foetal Calf Serum (FCS), 5 mls of l-Glutaimine, 5 mls of Penicillin/Steptomycin (10,000 U/ml and 10,000ug/ml respectively), 1.25mls of Fungizone. FCM without FCS contains an additional 50mls DMEM to compensate. Fibroblast cells were passaged when 90-100% confluent and used between passage numbers 5 and 9. Passaging of the fibroblasts was achieved using 1.5ml of a 1:1 mixture of 0.1% trypsin and 0.02% EDTA per T25 flask.

For investigation of human dermal fibroblast attachment and viability, fibroblasts were seeded at a density of 7.0×10^3 cells ml^{-1} . Human epidermal keratinocytes were seeded at a density of 3.8×10^5 cells/ml. Co-culture experiments used a keratinocyte seeding density of 1.5×10^5 cells/ml with irradiated dermal fibroblasts at 2×10^4 cells/ml, irradiated for 4780 seconds using a Caesium 137 sealed source. The attachment and

viability of the fibroblasts at three and seven days were assessed using an MTT-ESTA assay. This assay indicates viable cells and provides an indirect reflection of cell number, in that the cellular de-hydrogenase activity, which converts the MTT substrate to a coloured formazan product, normally relates to cell number. Cells were washed with 1ml of PBS solution and then incubated with 0.5 mg ml^{-1} of MTT in PBS for 40 minutes. 300µl of acidified Isopropanol was then used to elute the stain. 150µl was then transferred to a 96 well plate. The optical density was read using a plate reader set at a wavelength of 540nm with a protein reference of 630nm subtracted. In addition, the appearance of the cells was assessed and recorded at three and six or seven days.

The DNA content of the cells (which reflects cell number but not necessarily viability) was calculated at the same time periods using a Hoechst fluorescent stain (33258 Sigma Chemicals). Cells were incubated in 1ml of digestion buffer for 1 hour. This buffer consisted of 48g urea, which breaks up the cells and 0.04g of Sodium Dodecyl Sulfate (SDS), which protects the cells from DNAase, per 100ml of saline sodium citrate (SSC). Following digestion, cells were stained using the Hoechst fluorescent stain, in an SSC buffer at 1µg/ml. A fluorimeter was used to measure the fluorescence using excitation and emission wavelengths of 355 and 460nm respectively. A standard curve of known DNA concentrations was used to calculate the DNA content. For all experimental data presented, cells cultured on their own or in co-culture for six or seven days had a fresh change of media at day three.

In addition to culturing keratinocytes with fibroblasts under Green's standard media conditions (containing serum) we also cultured both cells on the PP surface in the absence of serum .Under these conditions fibroblast successfully supported keratinocyte proliferation to as great an extent as they did in the absence of serum.

EXAMPLES 1-3

In these examples, plasma polymerisations were carried out in a cylindrical reactor vessel (of 8 cm diameter and 50 cm in length), evacuated by a two stage rotary pump.

Plasma was sustained by a radio frequency (13.56 MHz) signal generator and amplifier inductively coupled to the reactor vessel. The base pressure in the reactor was 3×10^{-3} mbar.

Acrylic acid and 1,7-octadiene were co-polymerised at a plasma power of 2 W and a total flow rate of 2.0 sccm. Plasma co-polymers (PCP) were deposited onto a carrier polymer, polyhydroxybutyrate (Goodfellow, Cambridge, UK) and clean aluminium foil (for XPS analysis). The pressure during co-polymerization was typically 4.0×10^{-2} mbar.

For further experiments, selected polymerization conditions (100% acrylic acid, plasma power 1 W, polymerization pressure = 4.0×10^{-2} mbar) were used to deposit plasma polymers (PPs) onto a variety of carrier polymers, all of which were supplied as medical grade materials.

The polymer films used in Examples 1-3 are detailed in Table 1.

For all co-polymerisations, a deposition time of 20 minutes was used. The monomer mixtures were allowed to flow for a further 20 minutes after the plasma was switched off. This was done in an attempt to minimize the uptake of atmospheric oxygen by the deposits on exposure to the laboratory atmosphere.

In Examples 1-3, normal human adult keratinocytes (obtained from breast reductions and abdominoplasties), isolated from the dermal/epidermal junction (as previously described)[4], were used. These cells were cultured in complete Green's media (Hams F10) which included cholera toxin (0.1 nM), hydrocortisone (0.4 μg/ml), adenine (1.8×10^{-4} M), triiodo-1-thyronine (2×10^{-7} M), insulin (5 mg/ml), transferring (5 μg/ml), glutamine (2×10^{-3} M), fungizone (0.625 μg/ml), penicillin (1000 IU/ml), streptomycin (1000 μg/ml) and 10% fetal calf serum. Cells were cultured at 37°C, in a 5% CO_2 atmosphere.

Freshly isolated cells were used for cell culture experiments. Collagen coated carrier polymer samples were prepared by air drying a solution of collagen I (32 $\mu g/cm^2$) in a 0.1 M acetic acid (200 $\mu g/ml$) in a laminar flow cabinet overnight.

Cells were seeded at densities of 12.0×10^6 cells/ml onto triplicate of the surfaces using a 10 mm internal diameter stainless steel ring to keep the samples flat within a 6 well (3.5 cm diameter) tissue culture plate. After 24 hours in culture, the keratinocyte attachment on one sample from each triplicate was determined using an MTT-ESTA assay. This gives an estimate of viable cell number, the assay having previously been shown to parallel increases in cell number for human keratinocytes[5]. Cells were incubated with 0.5 mg ml^{-1} of MTT in PBS for 40 minutes. The stain was then eluted with acidified isopropyl alcohol. The optical density at 540 nm with a protein reference wavelength of 630 nm was then determined.

DED was prepared from a split thickness skin graft (STSG) from abdominoplasties and breast reductions. The method is described in detail elsewhere[6], but briefly, STSG were glycerol pretreated prior to ethylene oxide sterilization, using a standard Sterivit™ process using a mixture of 15% ethylene oxide and 85% carbon dioxide at a pressure of 5.5 atmospheres at 55°C to ensure a concentration of 1200mg ethylene oxide per litre in a sterilization cycle of 30 minutes. Sterilized skin was hydrated in a large volume of sterile phosphate buffered saline (PBS) for 24 hours at 37°C. Skin was then immersed in 1M NaCl solution for 6-8 hours at 37°C and the epidermis removed using sterile forceps. Skin was then rehydrated in an excess of keratinocyte culture medium (KCM) for 48 hours at 37°C prior to use.

The remaining two samples from each triplicate were placed in contact with DED and Green's media added so that the surface/DED junction sat at the air/liquid interface. The DED/surface wound bed models were placed in an incubator at 37°C for 4 days, after which the surfaces were separated from the DED and the level of cell transfer from surface to DED assessed using the MTT assay, as described above. MTT of the DED required that the DED was incubated with MTT for 120 minutes before elution of the

stain. After 4 days, a small sample of the DED was sent for hematoxylin-eosin (H&E) staining and histological evaluation. These samples were assessed for overall morphology, keratinocyte attachment to the dermis and extent of cell coverage.

The procedure detailed above was repeated for selected PPs deposited onto the eight medical grade polymers described in Table 1. All of these materials were ethylene oxide sterilized as outlined above, prior to the cell attachment and transfer experiments.

EXAMPLE 1

XP survey scan spectra of plasma copolymers (PCPs) prepared from acrylic acid and 1,7-octadiene revealed only carbon and oxygen in the deposits. The O/C ratios are shown in Table 2. The O/C ratio increased as the mole fraction of acrylic acid in the monomer feed increased. The C 1s core level spectrum of the PCP was peak fitted for various oxygen-containing functionalities. First, spectra were corrected for sample charging, setting the hydrocarbon signal to 285 eV. The following functionalities were then fitted: alcohol/ether (C-OH/R) at a shift of +1.5 eV; carbonyl (C=O) at +3.0 eV; carboxylic acid/ester (COOH/R) at +4.0 eV; and a β-shifted carbon bonded to carboxylate (C-COOH/R) at +0.7 eV. The results of peak fitting are shown in Table 2 and an example peak fit ($F_{aa}/F_{tot} = 1$) is shown in Figure 1. In the peak fit the FWHM of component peaks were kept equal and were in the range of 1.4 - 1.6 eV. The Gaussian to Lorentzian ratio (G/L) of the component peaks were also kept constant and were in the range 0.8 - 0.9. While XPS cannot distinguish between carboxylic acid and ester groups, grazing angle infra-red spectroscopy of plasma polymerised acrylic acid has shown, that at the low powers employed in this study, the carboxylate peak in the XP spectra can be assigned to carboxylic acid rather than ester[7]. Other carbon-oxygen functionalities present in the PCPs (besides carboxylic acid) including carbonyl and alcohol/ester. These arise as a result of fragmentation of the monomer in the plasma. Reaction between the deposit and water desorbed from the walls of the plasma vessel (during polymerisation) and atmospheric oxygen and water (after polymerisation) also contribute. The C-OH/R is thought to be predominantly hydroxyl. In a previous study we examined the identity of the oxygen-containing functionalities in PCPs of acrylic

acid/1,7-octadiene (prepared with varying molar fractions of acrylic acid in the monomer feed) in more detail[5]. Based on this study, we believe that on the PCP surface, keratinocytes respond to the carboxylic acid functionality, and not C-OH. The latter has to be present in high concentrations (25%) to promote cell attachment[8].

XPS on post-ethylene oxide sterilized samples revealed less than 20% change in the atomic % of acid at the PP surface compared with pre-ethylene oxide sterilised samples. . A comparison of a C 1s core line peak fit for an as-prepared and ethylene oxide sterilized sample comprising 100% acrylic acid is shown in Table 3.

EXAMPLE 2

After isolation of the keratinocytes, they were seeded onto the various surfaces and, after 24 hours, the surfaces were examined for viable cells using an MTT-ESTA assay. The results are shown in Figure 2. The data show that acid containing surfaces incorporating 50% and 100% acrylic acid in the monomer flow performed as well as collagen I, whilst keratinocyte attachment on the hydrocarbon surface was poor.

Cells attached to 100% acrylic acid PPs on a number of carrier polymers after ethylene oxide sterilization to the same degree as collagen I coated polymers (Table 4). Collagen I is a preferred substratum for the attachment of keratinocytes. The carrier polymers alone (i.e. no PP coating deposited) did not support cell attachment.

EXAMPLE 3

When the PP coated carrier polymer surfaces were separated from the DED the collagen I and 100% acid surfaces were well adhered to the DED, indicating that substantial transfer of keratinocytes from the surface to the DED had occurred. Surfaces with lower amounts of acid in the monomer flow were less well adhered, whilst the hydrocarbon surface readily peeled apart from the DED, suggesting a lesser degree of cell transfer had taken place.

The extent of cell transfer was assessed by staining of residual cells on the carrier polymer surfaces and by staining of keratinocytes transferred to the DED using the MTT-ESTA assay for both.

Figure 3 shows the results of an MTT assay on the surfaces after transfer of cells to the DED. The optical density values seen were extremely low (<0.02) compared to the initial attachment data (Figure 2).

Conversely, Figure 4 shows the results of the MTT assay on the DED where much higher MTT values were seen. Cells initially cultured on collagen I showed the highest presence on the DED. However, all cells cultured on acid surfaces showed some transfer to the DED indicative of proliferative keratinocytes. Transfer was most effective from the 100% acid surface (i.e. 100% acrylic acid in the plasma gas). Cells initially cultured on the hydrocarbon coated surface resulted in very little transfer of metabolically active cells.

Photographs in Figure 5 illustrate the appearance of the DED post-staining for metabolically active cells where it can be seen that cells initially cultured on collagen I (Fig. 5a) showed the greatest transfer to the DED, cells cultured on a hydrocarbon PP surface showed little transfer (Fig. 5b) and cells cultured on the acid surfaces (Fig. 5c) showed a good level of transfer - in some cases, approximating 80% of that seen for cells coated on collagen I. This experiment was repeated in triplicate with similar results.

A range of carrier polymers (Table 1) were plasma coated with acrylic acid (100% acrylic acid in the plasma gas). Keratinocytes transferred successfully to DED from all these surfaces. The extent of cell transfer was not affected by the ethylene oxide sterilization (Table 4).

Figure 6 shows the appearance of the epidermal layer formed after 4 days post-transfer of cells from (a) Collagen I, (b) 100% acrylic acid PP and (c) 50% acrylic acid PP

surfaces. H&E staining showed that keratinocytes transferred from a Collagen I surface were closely attached to the basement membrane of the DED (Fig. 6a). The presence of the dark nuclei in these proliferating cells can be clearly seen. Above these basal keratinocytes there is a pink keratin layer of differentiated cells. Similar cell morphology was observed for keratinocytes transferred from a 100% acrylic acid PP (Fig. 6b). Both Collagen I and 100% acrylic acid surfaces promoted extensive dermal coverage of DED (Figs. 6a and b respectively). Keratinocytes transferred from the 50% acrylic acid PP surface (Fig. 6c) shared the same morphological features as the Collagen I and 100% acrylic acid samples (i.e. basal keratinocytes attached to the dermis, nucleated cells within the epidermal layer). However, as demonstrated from the MTT-ESTA assays in Fig. 4, the number of transferred cells from the 50% acrylic acid surface was lower compared to the 100% acid surface, resulting in a less continuous keratin layer at the outer surface (Fig. 6c).

The use of a hydrocarbon plasma polymer as a negative control is important because a previous study has raised doubts about the suitability of TCPS as a control[9]. These concerns have arisen because of the surface treatments given to TCPS during manufacture, which may render TCPS unstable to aqueous solutions depending on the level of oxidation at the surface. It is unclear whether different batches of TCPS receive precisely the same amount of surface oxidation, or if this surface oxidation is susceptible to ageing.

Although the dependence of cell attachment on functional group concentration is yet to be fully explored, keratinocytes have shown enhanced attachment on surfaces with low amounts of acid functionality[1]. However, it should be recalled that the acid PCPs also contain other O-C functional groups, predominantly C-OH. Even so, our previous studies have demonstrated that acid PCPs are comparable to collagen I in terms of degree of confluency and cell number (as determined by DNA assay).

In aqueous media, the acid PCPs can hydrate, and the stability of acid PCPs has been shown to be dependent on the concentration of acrylic acid in the monomer flow. High

concentrations of acid (>60% of the total flow) result in less stable surfaces. This requirement led to the development of low concentration acid surfaces (<5%) as being ideal for promoting attachment and subsequent proliferation. However, with regard to cell transfer from acid surfaces, different criteria are likely to apply. Whilst low concentrations of acid groups impart stability to the surface, the keratinocytes may be sufficiently well attached that transfer is inhibited. This assertion is borne out in the results of the transfer experiments, where 25% acid flow in the monomer feed (equivalent to 2.6% carboxylic acid at the PCP surface) showed the lowest degree of transfer to DED. In contrast, with 100% acid flow in the monomer (>20% carboxylic acid at the PP surface), transfer of cells was significantly higher. These surfaces were only out-performed by collagen I. With 50% acid in the monomer flow, transfer was intermediate between the high and low functionality surfaces, as would be expected. These results indicate that the optimum surfaces for promoting attachment and proliferation may not be those which result in the largest degree of keratinocyte transfer from PCPs to DED. The low amount of transfer from the hydrocarbon PP confirm that such a surface is not capable of supporting keratinocytes in a proliferative state. Although the dependence of cell transfer on functional group concentration is yet to be fully explored, keratinocytes show enhanced transfer from surfaces with high amounts of acid functionality. It is clear, therefore, that there exists a compromise between surfaces which promote proliferation (low acid functionality) and those which promote transfer (high acid functionality).

The results of the attachment and transfer experiments show that the choice of carrier polymer does not greatly affect the cell behaviour, and that the 100% acrylic acid PP surfaces are sufficiently durable to withstand ethylene oxide sterlization. These PP surfaces are also unaffected by γ-irradiation, but the use of PVC in the carrier polymer precludes this technique from being used, unless the PVC has been stabilised for γ-irradiation. The degradation of PVC under γ-irradiation is well documented[10-12].

In comparison to normal skin[13], the transferred cells in the current study did not always achieve a normal pattern of keratinization. Typically the variations seen were (i) the presence of nucleated cells within the superficial layers of the epidermis, similar to the

parakeratosis seen in dysmorphic skin conditions, and (ii) small aggregates of cells with a similar morphology to basal keratinocytes present in the superficial epidermis (Figs. 6a, b and c). However, the DED comprising keratinocytes transferred from Collagen I and 100% acrylic acid surfaces (Figs. 6a and b respectively) showed a pattern of keratinization resembling normal skin in a number of features, and it is seen that keratinocytes appeared to grow into the clefts and spaces left by features such as hair follicles and glandular structures of the skin. Both the Collagen I and 100% acrylic acid surfaces promoted extensive keratinocyte coverage of the DED, typified by a continuous epidermal layer of cells. One distinction between the Collagen I (Fig. 6a) and 100% acrylic acid (Fig. 6b) is that the epidermal keratinocytes from the latter surface exhibit some separation from the basal keratinocytes. The majority of the epidermal keratinocytes are, however, in contact with the proliferating cells below.

In serum-containing medium, cells respond to an absorbed layer of protein, rather than directly to the substratum itself[14]. This interfacial protein layer absorbs (almost) spontaneously. The differences in cell response to the substrata under investigation suggest that there are either changes in the composition of the protein films that adsorb or in the activities of these proteins after adsorption, or a combination of both of these. Cell attachment has been shown to be supported by a number of adhesive proteins, such as fibronectin and vitronectin. Tidwell et al.[15] have shown differences in the protein layers that develop on SAMs with alkanethiolates of different terminal chemistries and that these, in turn, support different levels of bovine aortic endothelial cell attachment.

Whilst cell attachment and spreading are important conditions for cell proliferation, they are not exclusive conditions. Serum is also a source of growth factors and these have been shown to be essential for the proliferation of primary mammalian cells. It has been suggested that the adsorption of growth factors onto extracellular matrix material plays a role in their activation[16].

Previously, keratinocytes have been delivered to wound beds in vitro and in vivo using fibrin glue[17] and polymer sheets[(18)]. These have relied upon initial keratinocyte attachment to an animal-derived material (e.g. collagen). The risk of disease

transmission is a growing concern in the clinical use of cells. Therefore, reduced reliance on animal-derived materials is seen as important. The plasma polymerized surfaces described herein effectively promote cell attachment and transfer to a degree sufficient for consideration for clinical use.

Based upon the above discussion, it is evident that the success of the acid PCPs in supporting keratinocyte attachment and transfer is multi-factorial. However, our results would indicate that keratinocyte attachment and transfer are promoted specifically by the carboxylic acid functionality. This is most probably through control of the interfacial protein layer that forms from serum.

Plasma polymerized surfaces containing high concentrations of carboxylic acid groups encouraged keratinocyte attachment and transfer to DED compared to hydrocarbon surfaces. The initial attachment of cells on surfaces containing ~20% acid groups was comparable to that of cells on collagen I substrates after 24 hours in culture.

EXAMPLE 4

From medical grade PVC, (supplied in transparent, conformable rolls), 6cm discs were punched out and exposed to plasma polymerisation using an acrylic acid monomer. Deposition was carried out under the following conditions: acrylic acid flow rate of 12 sccm using a plasma power of 1W for a period of 20 mins in a tubular glass reactor. After 20 mins the plasma was turned off and the acrylic acid was left flowing through the reactor for 20 minutes before the samples were exposed to laboratory air. This minimises the uptake of oxygen into the plasma polymer layer from the laboratory atmosphere. The samples were sterilised either by ethylene oxide or gamma irradiation before clinical use. The surface thus produced is a TranCell^{tm} surface.

The ulcers shown in Figures 7a-i resulted from diabetes and caused by neuropathy. The examples are from 3 patients. The wounds were non-infected and and debrided before before delivery of the patients cells from the treated PVC substrate. Other patients were treated in a similar fashion to treat non-healing wounds, pretibial lacerations and wounds which were unsuitable for grafting with similar results.

In all examples a biopsy of 2 x 1.5cm in diameter was obtained from a patient as a thin shave. The patients keratinocytes were then expanded in the laboratory and the majority frozen down. Cells were seeded onto plasma polymer surfaces for 2 days before applying the cells back to the patients wound.

The examples shown in Figures 7a-i illustrate how repeated applications of the substrate according to the invention can produce remarkable healing in chronic skin wounds.

EXAMPLE 5

As in Example 3 , transfer of the cells from a 100% acid surface (i.e. 100% acrylic acid in the plasma gas) was measured by three different wound beds *in vitro*. The 100% acid surface is the TranCell™ dressing. The results show that the degree of transfer is effected by the presence/absence of a basement membrane (BM) in the wound bed substrate. The highest degree of transfer (TranCell, collagen I) occurred with the BM present. These data are suggestive of the fact that during the early stages of application of the substrate, healing is promoted by keratinocytes attached to the plasma polymerised surface which secrete growth factors.

REFERENCES

1. Haddow DB, France RM, Short RD, MacNeil S, Dawson RA, Leggett GJ, Cooper R. Comparison of proliferation and growth of human keratinocytes on plasma co-polymers of acrylic acid/1,7-octadiene and self-assembled monolayers. J Biomed Mater Res 1999;47(3):379-387.
2. Dawson RA, Goberdhan NJ, Freedlander E, Mac Neil S. Influence of extracellular matrix proteins on human keratinocyte attachment, proliferation and transfer to a dermal wound model. Burns 1996;22:93-100.
3. Beamson G, Briggs D. High Resolution XPS of Organic Polymers: The Scienta ESCA300 Handbook. John Wiley and Sons; 1992. p230.
4. Goberdhan NJ, Edgecombe M, Freedlander E, MacNeil S. Extracellular matrix proteins induce changes in intracellular calcium and cyclic AMP signalling systems in cultured human keratinocytes. Burns 1997;23(2):122-130.

5. France RM, Short RD, Dawson RA, MacNeil S. Attachment of human keratinocytes to plasma co-polymers of acrylic acid/1,7-octadiene and allyl amine/1,7-octadiene. J Mater Chem 1998;8(1):37-42.

6. Chakrabarty KH, Dawson RA, Harris P, Layton C, Babu M, Gould L, Phillips J, Leigh I, Green C, Freedlander E, MacNeil S. Development of autologous human dermis-epidermis composites based on a sterilised human allodermis for clinical use. Brit J Dermatol 1999;141(5):811-823.

7. O'Toole L, Beck AJ, Short RD. Characterisation of plasma-polymers of acrylic acid and propanoic acid. Macromolecules 1996; 29(15):5172-5177.

8. France RM, Short RD, Duval E, Dawson RA, MacNeil S. Plasma co-polymerisation of allyl alcohol/1,7-octadiene: surface characterisation and attachment of human keratinocytes. Chem Mater 1998;10(4):1176-1183.

9. France RM, Short RD. Plasma treatment of polymers - effects of energy transfer from an argon plasma on the surface chemistry of poly(styrene), low density poly(ethylene), poly(propylene) and poly(ethylene terephthalate). J Chem Soc Faraday Trans. 1998;93(17):3173-3178.

10. Wang Q, Nagy S. Improving gamma-radiation stability of PVC-A review. Journal of Vinyl and Additive Technology. 1999: 5(1): 4-11.

11. Torikai A, Hasegawa H. Accelerated photodegradation of poly(vinyl chloride). Polym. Degrad. Stabil. 1999: 63(3): 441-445.

12. Bacaloglu R, Fisch M. Degradation and stabilization of poly(vinyl chloride). %. Reaction mechanism of PVC degradation. Polym. Degrad. Stabil. 1995: 47(1): 33-57.

13. Ghosh MM, Boyce S, Layton C, Freedlander E, MacNeil S. A comparison of methodoligies for the preparation of human epidermal-dermal composites. Annals of Plastic Surgery 1997;39(4):390-404.

14. Griesser HJ, Chatelier RC, Gengenbach TR, Johnson G, Steele JG. Growth of human cells on plasma polymers-putative role of amine and amide groups. J Biomater Sci Polym Ed 1994;5:531-554.

15. Tidwell CD, Ertel SI, Ratner BD, Tarasevich BJ, Artre S, Allara DL. Endothelial cell growth and protein adsorption on terminally functionalised self-assembled monolayers of alkanethiolates on gold. Langmuir 1997;13(13):3404-3413.

16. Wight TN, Kinsella MG, Qwarnstrom EE. The role of proteoglycans in cell adhesion migration and proliferation. Ann Rev Cell Biol 1992;8:365-393.

17. Kaiser HW etal., Cultured Autologous keratinocytes in fibrin glue suspension, exclusively and combined with STS-allograft (preliminary clinical and histological report of a new technique) Burns, 1994, 20: 23-29

18. Grant I at al, Demonstration of epidermal transfer from a polymer neutrane using genetically marked porcine keratinocytes, Burns, 2001, 27: 1-8

CLAIMS

1. A cell transfer substrate comprising at least one surface wherein said surface is obtainable by plasma polymerisaton of a monomer preparation to provide a plasma coated surface.

2. A substrate according to Claim 1 wherein said surface is at least 2% acid.

3. A substrate according to Claim 1 wherein said surface is at least 5% acid.

4. A substrate according to any of Claims 1-3 wherein said surface has an acid content of 2 - 20%.

5. A substrate according to Claim 1 wherein said surface has an acid content greater than 20%.

6. A substrate according to any of Claims 1-5 wherein said surface has been treated by plasma polymerisation with a volatile acid.

7. A substrate according to Claim 6 wherein said volatile acid is selected from the group consisting of: acrylic acid; methacrylic acid; propanoic acid; or maleic anhydride.

8. A substrate according to Claim 1 wherein said surface has been treated by plasma polymerisation with a volatile alcohol.

9. A substrate according to Claim 1 wherein said surface has been treated by plasma polymerisation with a volatile amine.

10. A substrate according to Claim 1 wherein said surface has been treated by plasma polymerisation with a mixture of volatile acid and volatile hydrocarbon.

11. A substrate according to Claim 1 wherein said substrate is coated with a plasma co-polymer.

12. A substrate according to Claim 11 wherein said co-polymer is a plasma co-polymer of an acidic monomer.

13. A substrate according to any of Claims 1-12 wherein said substrate comprises an olefin or an aliphatic polyolefin polymer..

14. A substrate according to any of Claims 1-12 wherein said substrate comprises a polymer selected from the group consisting of: diene rubber; butyl rubber; nitrile rubber; polybutadiene; polyisoprene ethylene-propylene rubber; or polychlorprene.

15. A substrate according to any of Claims 1-12 wherein said substrate comprises a polymer which is an ethylene co-polymer.

16. A substrate according to any of Claims 1-12 wherein said substrate comprises a vinyl polymer.

17. A substrate according to Claim 16 wherein said vinyl polymer is selected from the group consisting of: polyvinyl chloride; polyvinyl acetate; or polyvinyl alcohol.

18. A substrate according to any of Claims 1-12 wherein said substrate comprises an acrylic polymer.

19. A substrate according to any of Claims 1-12 wherein said polymer comprises a styrene based plastic.

20. A substrate according to any of Claims 1-12 wherein said polymer comprises a polyamide.

21. A substrate according to any of Claims 1-12 wherein said polymer comprises a polymer wherein said polymer is a silicone polymer.

22. A substrate according to any of Claims 1-21 and at least one mammalian cell attached thereto.

23. A substrate according to Claim 22 wherein said mammalian cell is human.

24. A substrate according to Claim 23 wherein said human mammalian cell is selected from the group consisting of: epidermal keratinocytes; dermal fibroblasts; adult skin stem cells; embryonic stem cells; melanocytes, corneal fibroblasts, corneal epithelial cells, corneal stem cells; intestinal mucosa fibroblasts, intestinal mucosa keratinocytes,oral mucosa fibroblasts,oral mucosa keratinocytes, urethral fibroblasts and epithelial cells, bladder fibroblasts and epithelial cells, neuronal glial cells and neural cells, hepatocyte stellate cells and epithelial cells.

25. A substrate according to Claim 24 wherein said skin cell selected from the group consisting of: a keratinocyte; a fibroblast; an adult skin stem cell; or a melanocyte.

26. A method to culture mammalian cells on a substrate comprising the steps of:

i) providing a preparation comprising;
   a) mammalian cells;
   b) a substrate according to any of Claims 1-21;
   c) cell culture medium sufficient to support the growth of said mammalian; and

ii) providing cell culture conditions which promote the proliferation of said mammalian cells on said substrate.

27. A method according to Claim 25 wherein said substrate has attached thereto, fibroblast feeder cells.

28. A method according to Claim 26 or 27 wherein said medium does not include serum.

29. A method according to any of Claims 26-28 wherein said mammalian cells are human.

30. A method according to Claim 29 wherein said mammalian cells are selected from the group consisting of: epidermal keratinocytes; dermal fibroblasts; adult skin stem cells; melanocytes, corneal fibroblasts, corneal epithelial cells, corneal stem cells; intestinal mucosa fibroblasts, intestinal mucosa keratinocytes,oral mucosa fibroblasts,oral mucosa keratinocytes, urethral fibroblasts and epithelial cells, bladder fibroblasts and epithelial cells, neuronal glial cells and neural cells, hepatocyte stellate cells and epithelial cells.

31. A method according to Claim 29 or 30 wherein said mammalian cells are autologous.

32. A method according to Claim 31 wherein said cells are keratinocytes.

33. A method according to any of Claims 26-32 wherein said fibroblast feeder cells are human.

34. A method according to Claim 33 wherein said fibroblast feeder cells are human dermal fibroblasts or human oral fibroblasts.

35. A method according to Claim 33 or 34 wherein said feeder cells are autologous.

36. According to a further aspect of the invention there is provided a method to treat a mammal, preferably a human, suffering from a wound comprising contacting the wound with a mammalian cell-bearing substrate according to any of Claims 21-25.

37. A method according to Claim 36 wherein said wound is a cutaneous wound.

38. A method to coat a substrate comprising the steps of:

i) providing at least one organic monomer;

ii) creating a plasma of said organic monomer; and

iii) coating said substrate with said plasma to provide a plasma coated substrate according to any of Claims 1-21.

39. A method according to Claim 38 wherein said monomer is an acid monomer source comprising 30-99% acid monomer.

40. A method according to Claim 39 wherein said acid monomer source consists of a 100% acid monomer source.

41. A method according to Claim 40 wherein said method consists of a 100% acrylic acid source.

42. A method to treat the substrate comprising the steps of:

i) providing a selected ratio of a monomer and a hydrocarbon in a gas feed;

ii) creating a plasma of said mixture;

iii) coating said substrate with said plasma to provide a plasma coated co-polymer substrate according to Claim 11 or 12.

43. A method according to Claim 42 wherein said monomer is an acid monomer.

44. A method according to Claim 43 wherein said acid is acrylic acid and said hydrocarbon is a diene.

45. A method according to Claim 44 wherein said diene is 1,7-octadiene.

46. A method according to any of Claims 42-45 wherein said plasma comprises 50-100% unsaturated acid and 0-50% diene in the gas feed.

47. A method according to Claim 46 wherein said plasma comprises the following ratios of acid and diene:

Acid	diene
%	%
50	50
60	40
70	30
80	20
90	10
100	0

in the gas feed.

48. A method according to Claim 47 wherein said substrate comprises a vinyl polymer.

49. A method according to Claim 46 or 47 wherein said acid is acrylic acid and said hydrocarbon is a diene.

Table 1

Polymer	Source
PVC	Baxter S.A. (Belgium), Cryocyte Bag
PVC	Baxter S.A. (Belgium), R2003, PL 1240, Transfer Pack
PVC	Baxter S.A. (Belgium), R2089, PL 146, Transfer Pack
PVC	Pall Medical (U.K.), WBT 410CU, Plasma Bag
Polypropylene	Solvay Draka (Neth.), Solmed Infuflex 7210
PVC	Solvay Draka (Neth.), Solmed Transfufol, 3226 Seta
PVC	Solvay Draka (Neth.), Solmed Vinylfoil, 5550 Seta Glad
PHB	Goodfellow (UK), Biopol

Table 2

		% functionality in C 1s core level			
F_{aa}/F_{tot}	O/C ratio	C-C, C-H	C-OH/R	C=O	COOH/R
0	0.04	95.8	4.7	-	-
0.25	0.11	88.4	5.7	1.0	2.6
0.5	0.16	87.1	1.4	1.2	5.4
1.0	0.51	50.1	5.8	2.9	20.6
Carrier	0.47	52.2	1.2	16.0	16.0

Table 3

Surface Functionality	Atomic % on as-treated PP	Atomic % on ethylene oxide sterilized PP
C-C/C-H	58.9	57.4
C-OH/R	2.5	10.8
C=O	3.6	2.9
COOH	18.3	15.3
O/C ratio	0.49	0.45

Table 4.

Polymer	Cell attachment	Cell transfer to DED	Rank (by eye, based upon MTT assay of DED)	Suitability for clinical handling
1) Pall Medical (UK), WBT 410CU, Plasma Bag (PVC)	+++	++	2	✓
2) Baxter, SA (Belgium), R2003, PL 1240, Platelet Bag (PVC)	+++	+++	1	✓
3) Baxter, SA (Belgium), R2089, PL 146, Transfer Pack (PVC)	++	+	5	✓
4) Baxter, SA (Belgium), Cryocyte Bag (silicone)	++	++	3	✓
5) Solvay Draka (Neth.), Solmed Infuflex 7210 (Polypropylene)	+++	++	2	x(not sufficiently pliable)
6) Solvay Draka (Neth.), Solmed Transfufol, 3226 Seta (PVC)	+++	+	4	x(too thick)
7) Solvay Draka (Neth.), Solmed Vinylfoil, 5550 Seta Glad, rough side (PVC)	+++	++	1	✓
8) Goodfellow (UK), Biopol (PHB)	+++	+++	1	✓

5

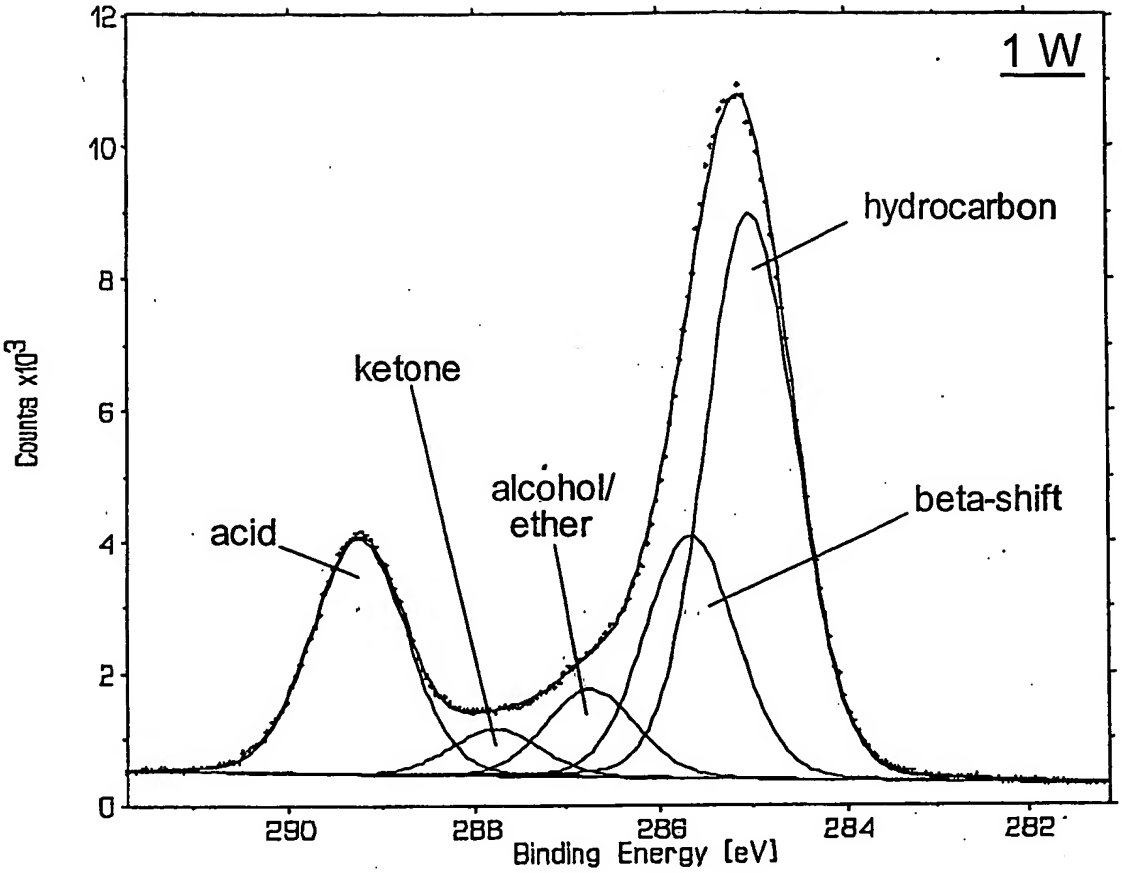

1 W
hydrocarbon
ketone
alcohol/
ether
beta-shift
acid
Counts x10^3
Binding Energy [eV]
12
10
8
6
4
2
0
290
288
286
284
282

Figure 1

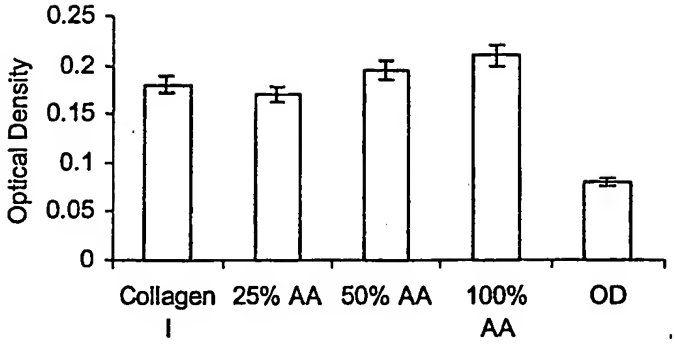

0.25
0.2
0.15
0.1
0.05
0
Optical Density
Collagen I
25% AA
50% AA
100% AA
OD

Figure 2

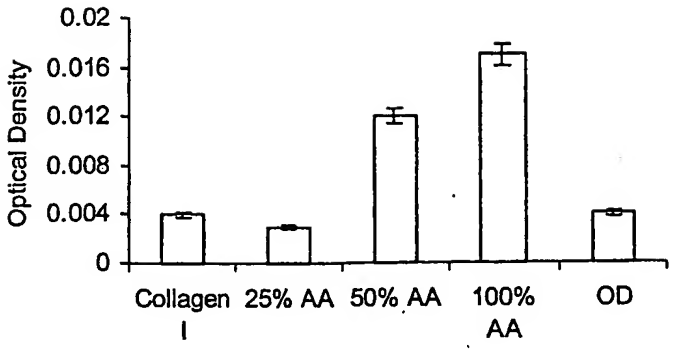

0.02
0.016
0.012
0.008
0.004
0
Optical Density
Collagen I
25% AA
50% AA
100% AA
OD

Figure 3

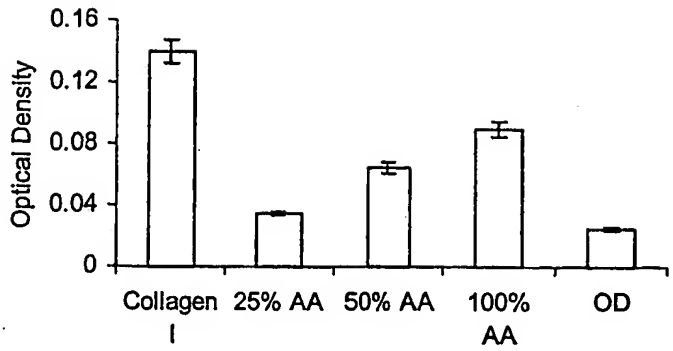

0.16
0.12
0.08
0.04
0
Optical Density
Collagen I
25% AA
50% AA
100% AA
OD

Figure 4

5

Figure 5a

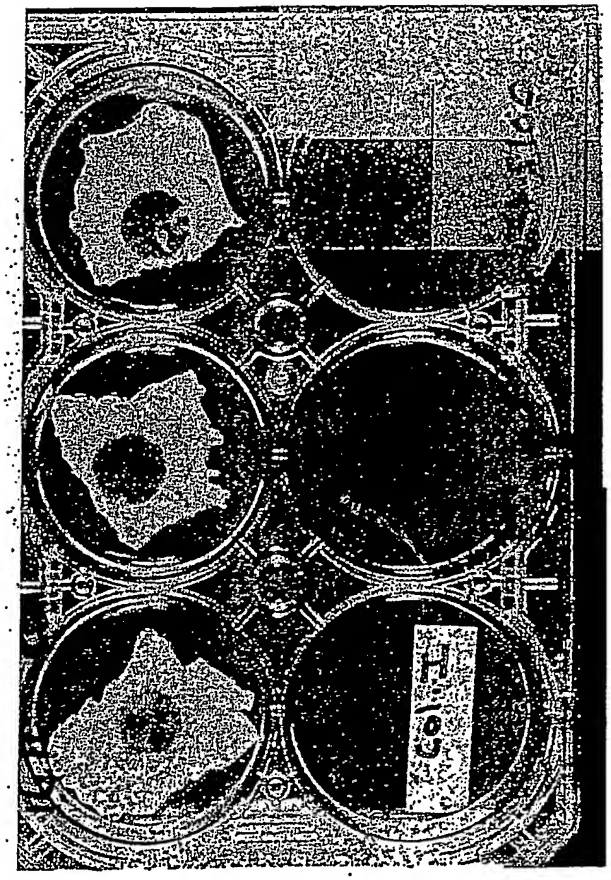


Figure 5b

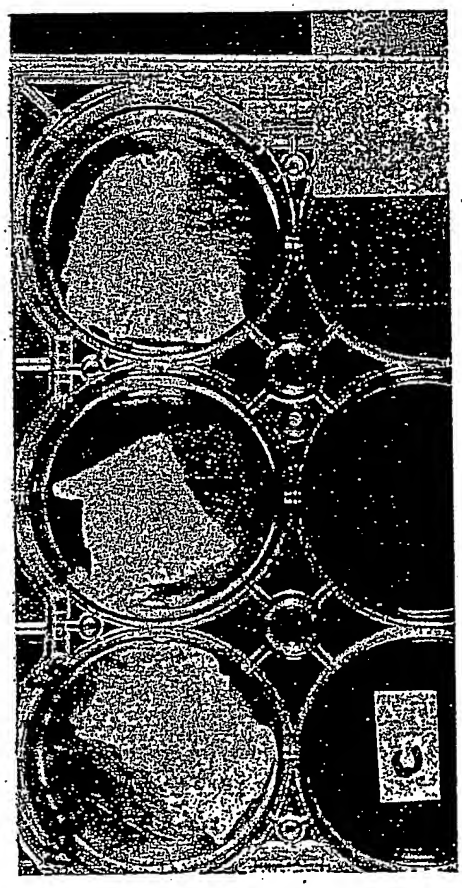
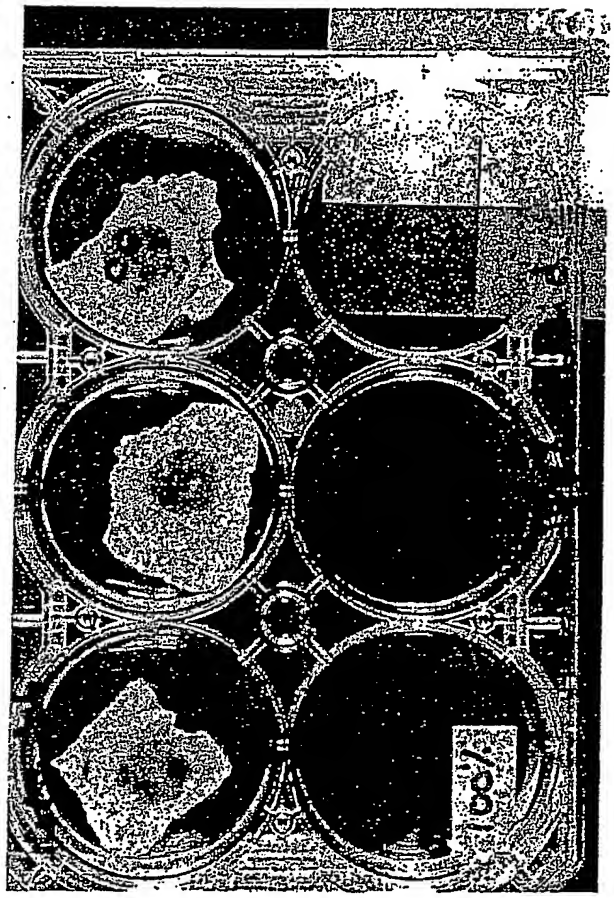


Figure 5c



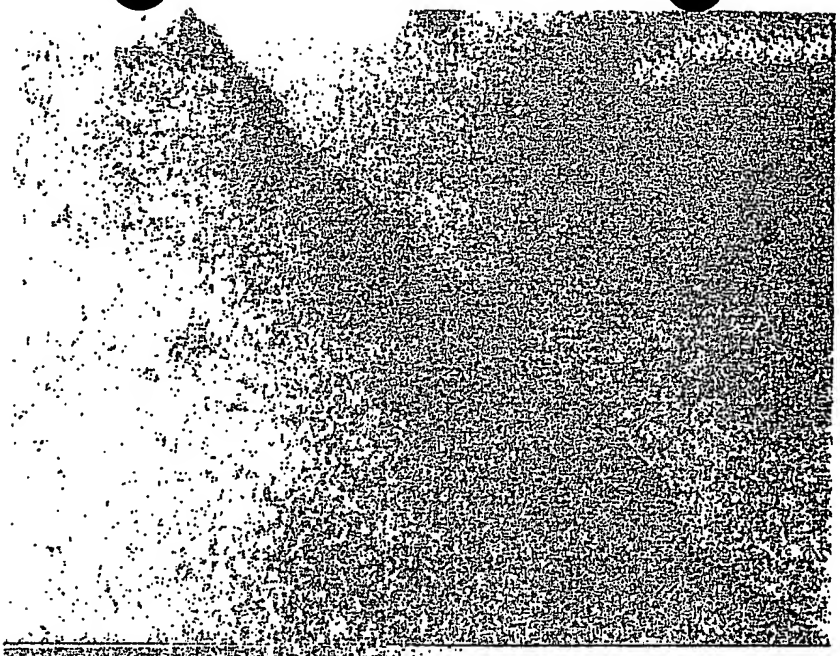


Figure 6a

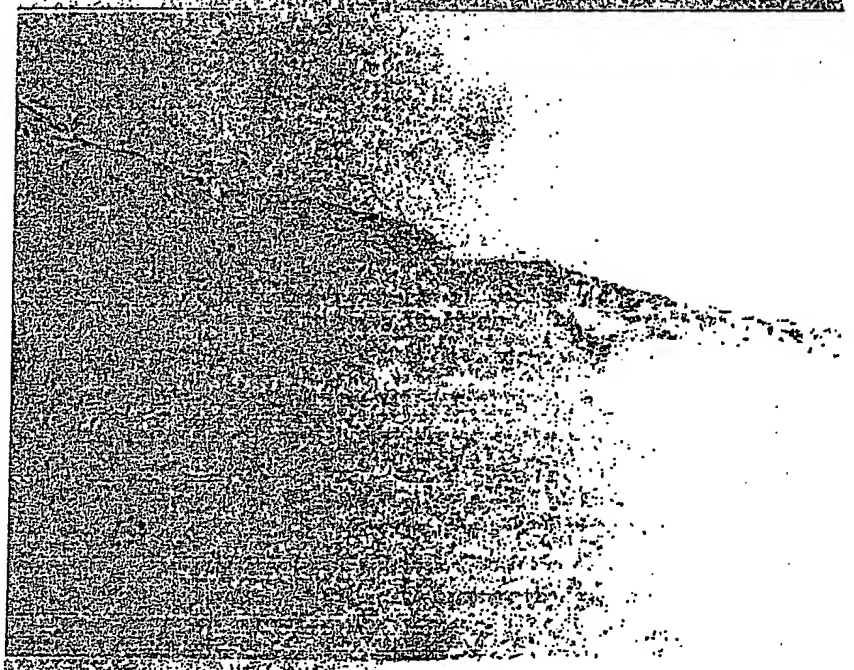


Figure 6b

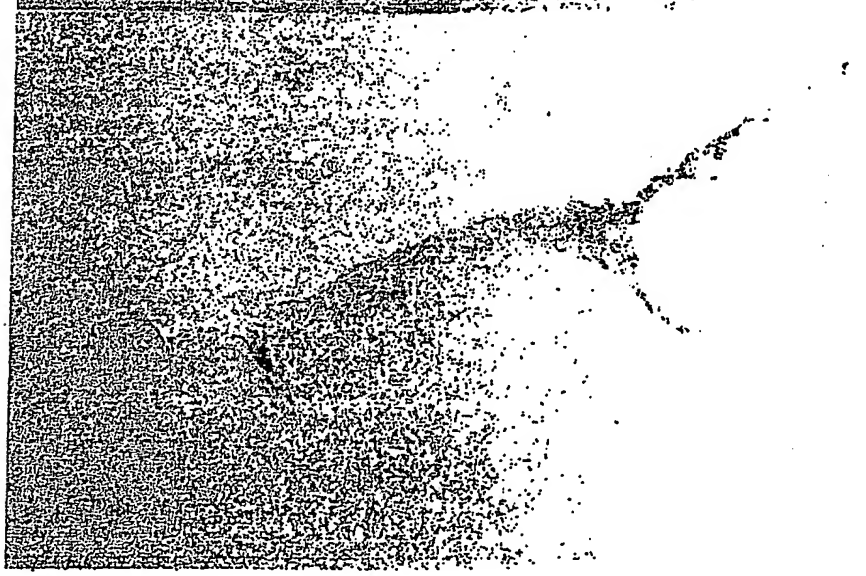


Figure 6c

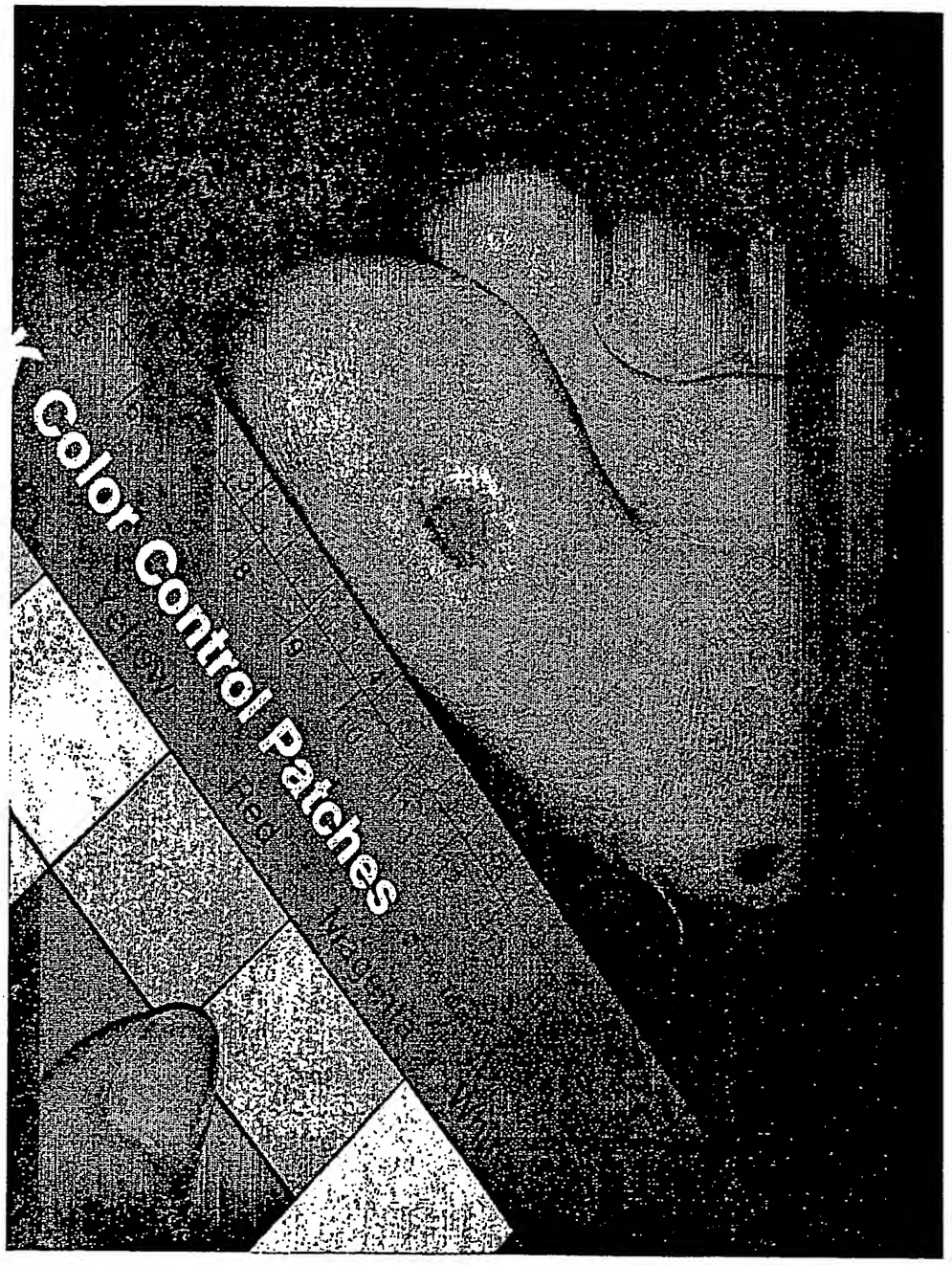


Figure 7a Patient 2: Before treatment

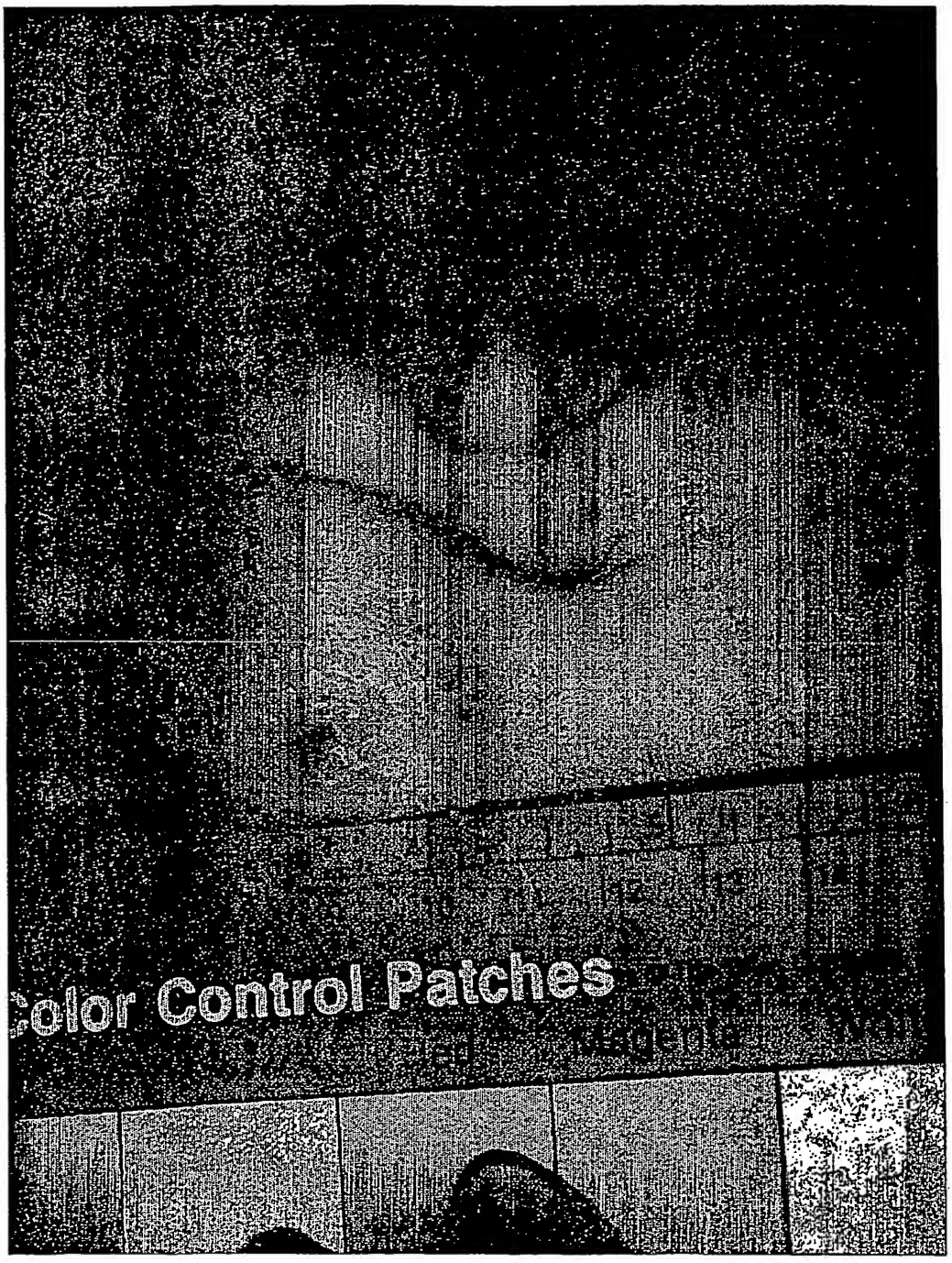


Figure 7b Patient 2: After 8 applications

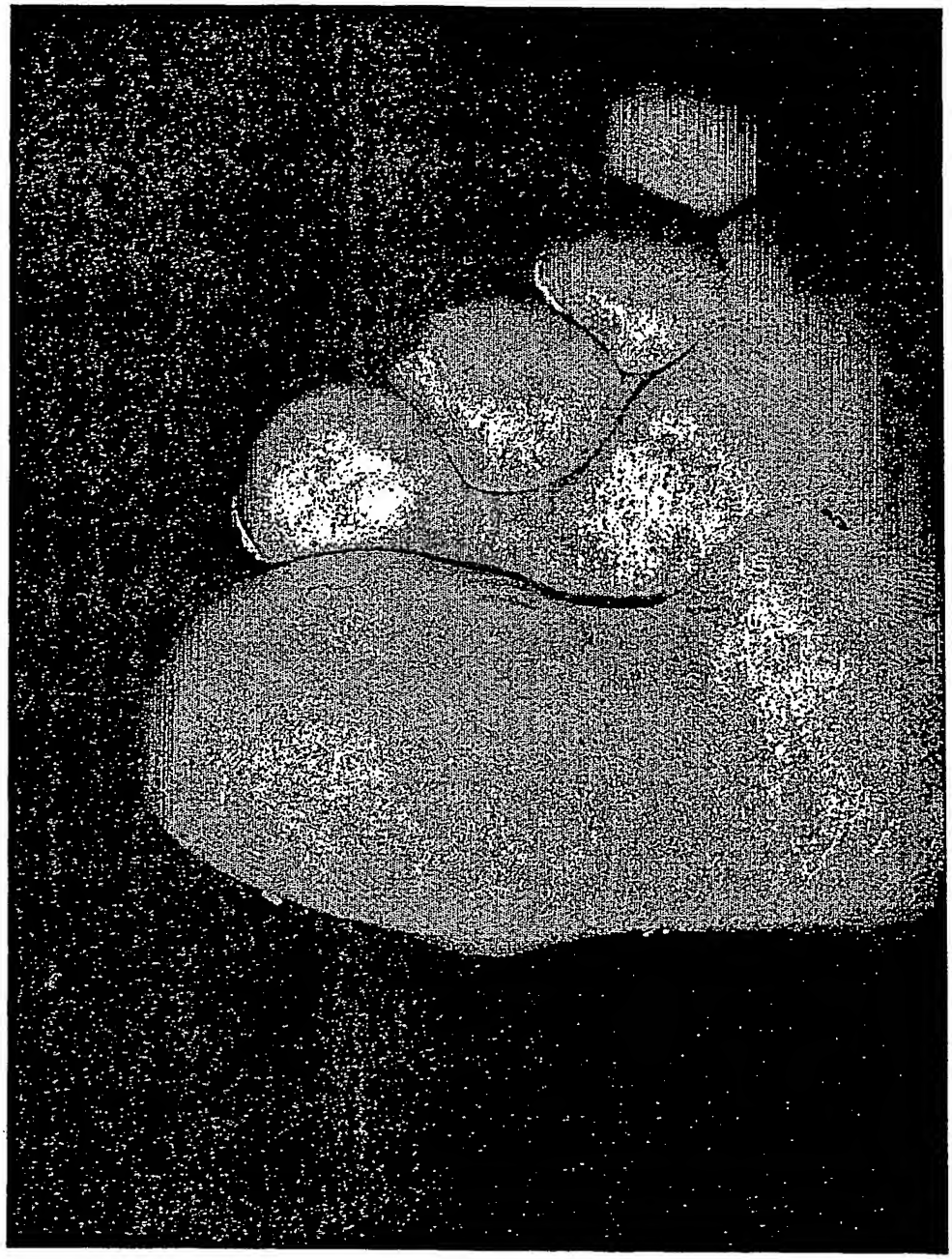


Figure 7c Patient 2: After 5 month follow up

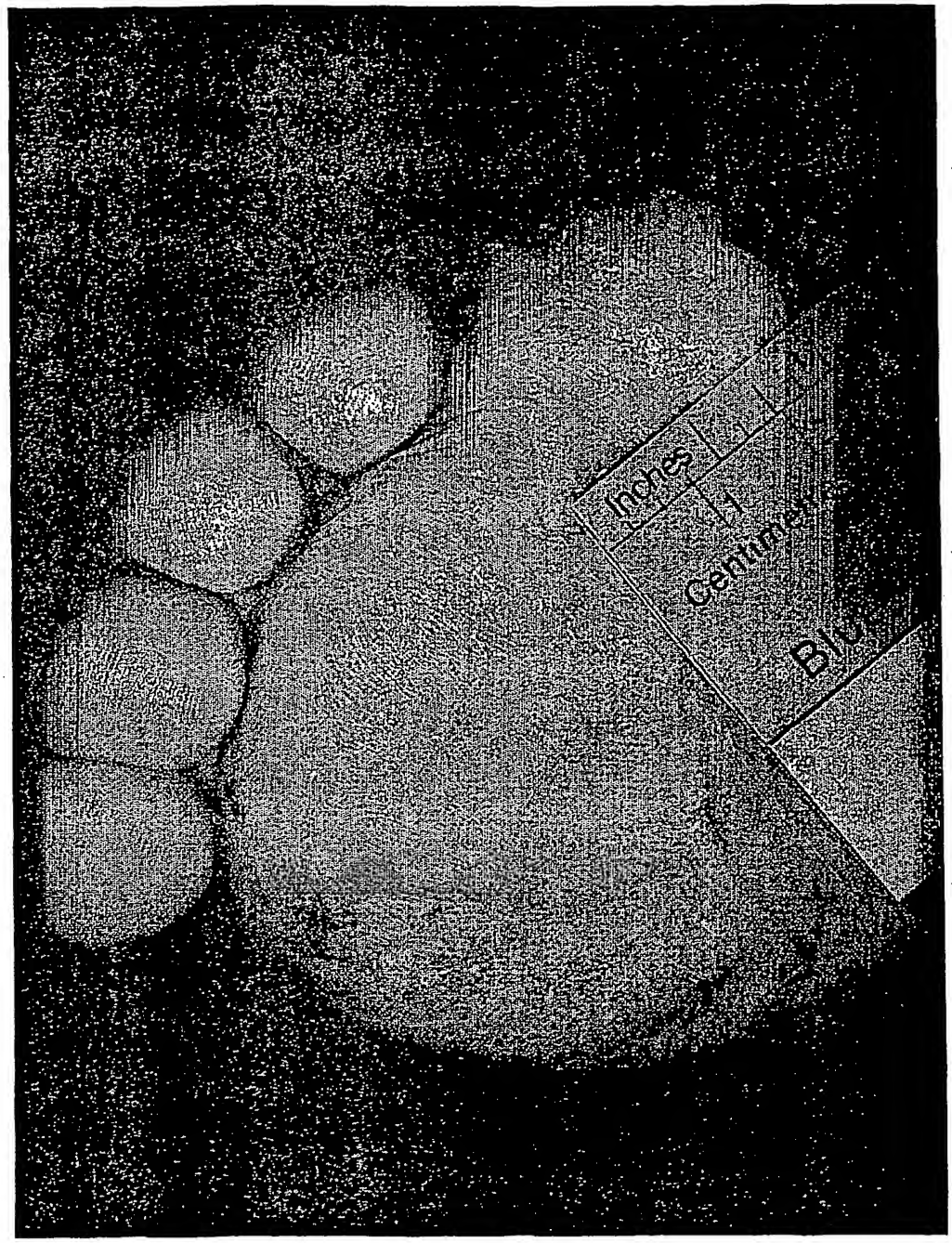


Figure 7e Patient 3: After 2 applications

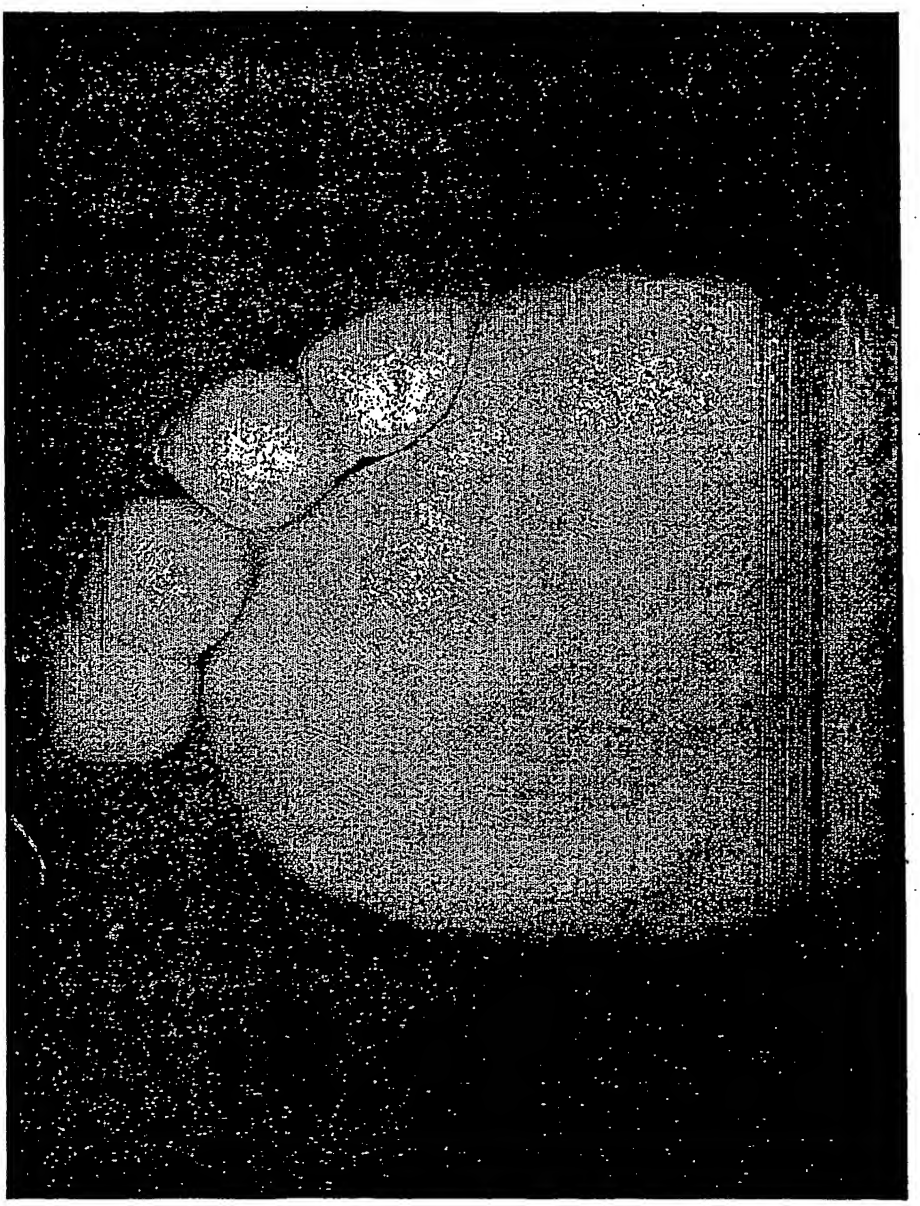


Figure 7f Patient 3: After 1 month follow up

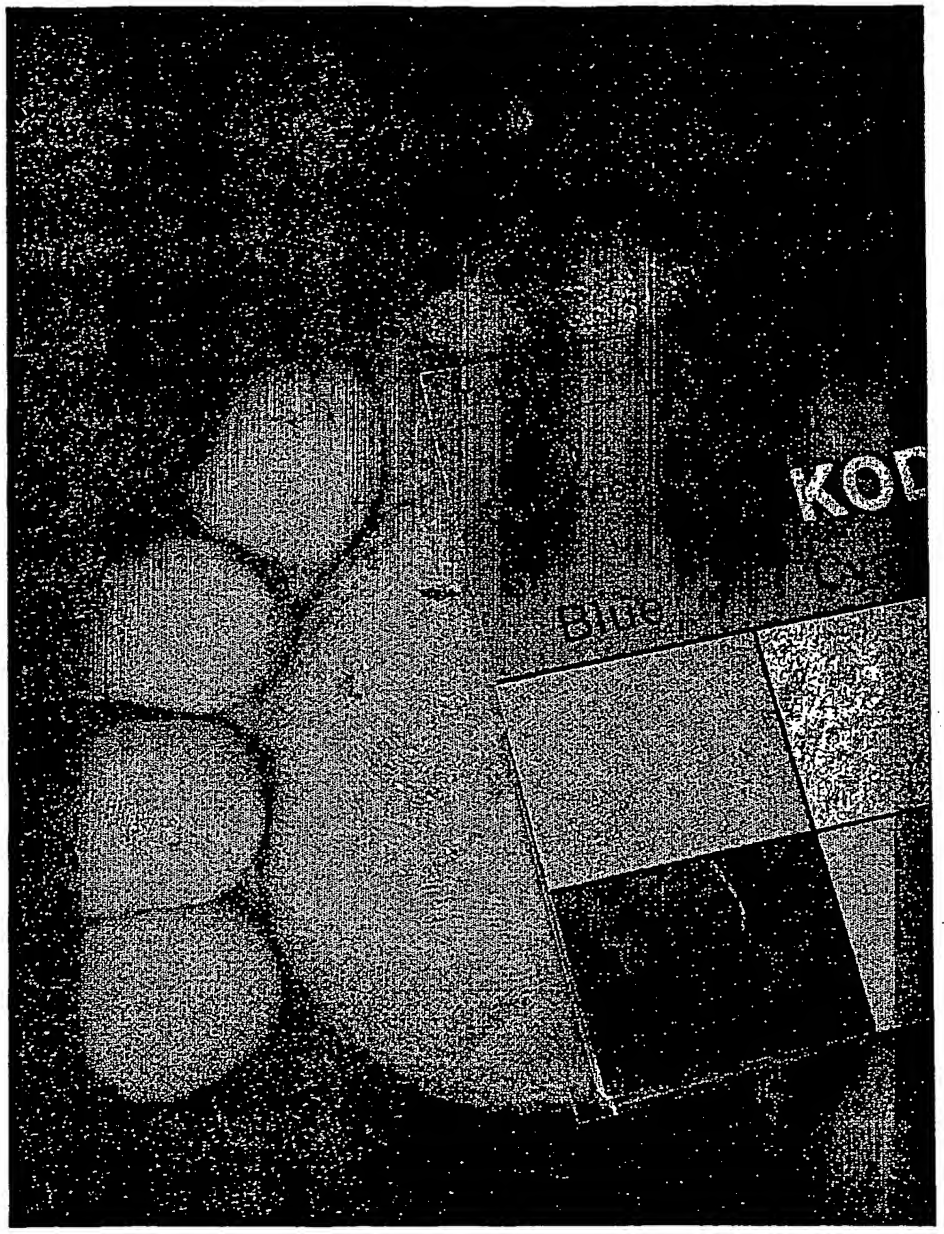


Figure 7g Patient 3: Before treatment

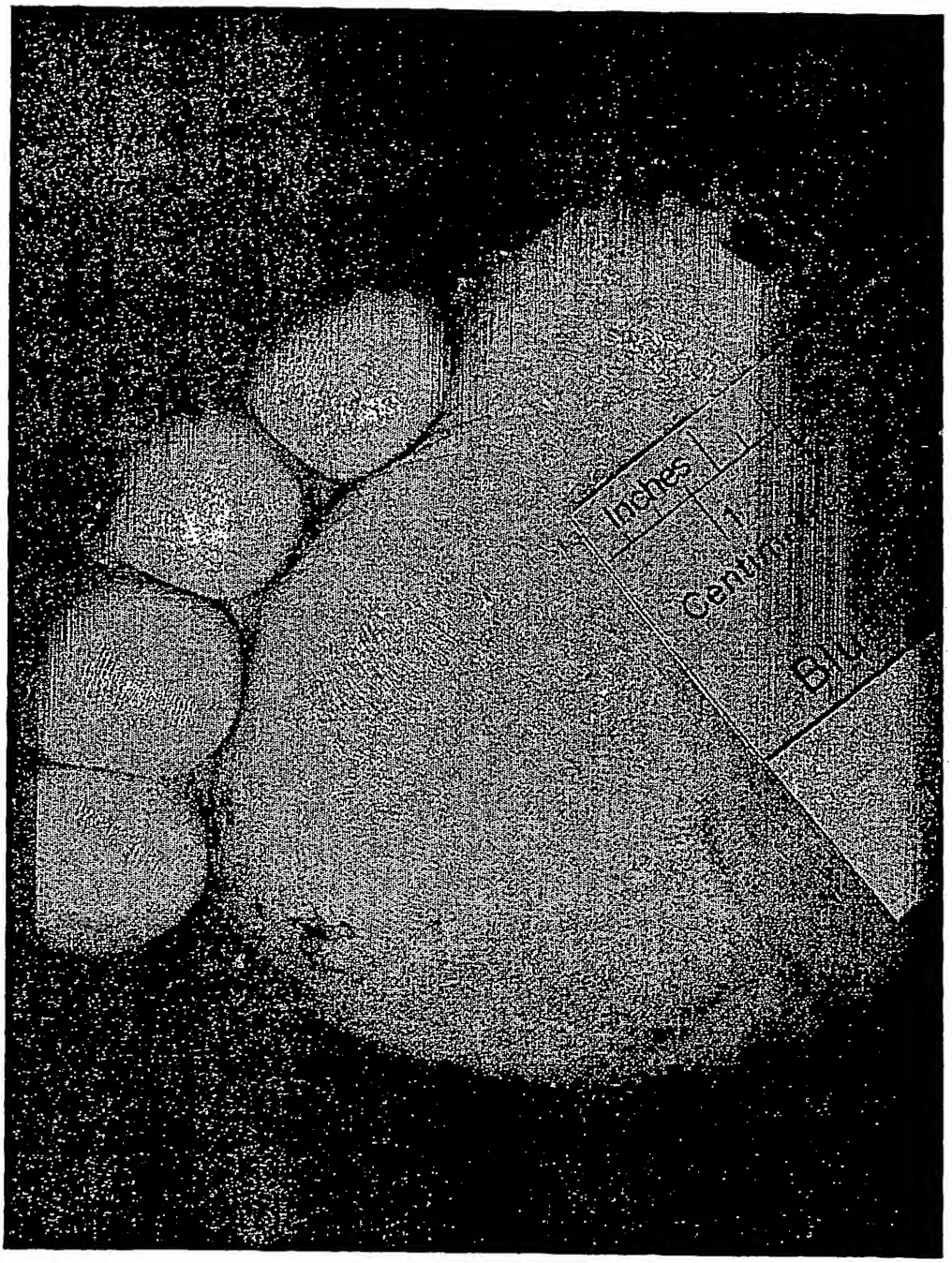


Figure 7h Patient 3: After 2 applications

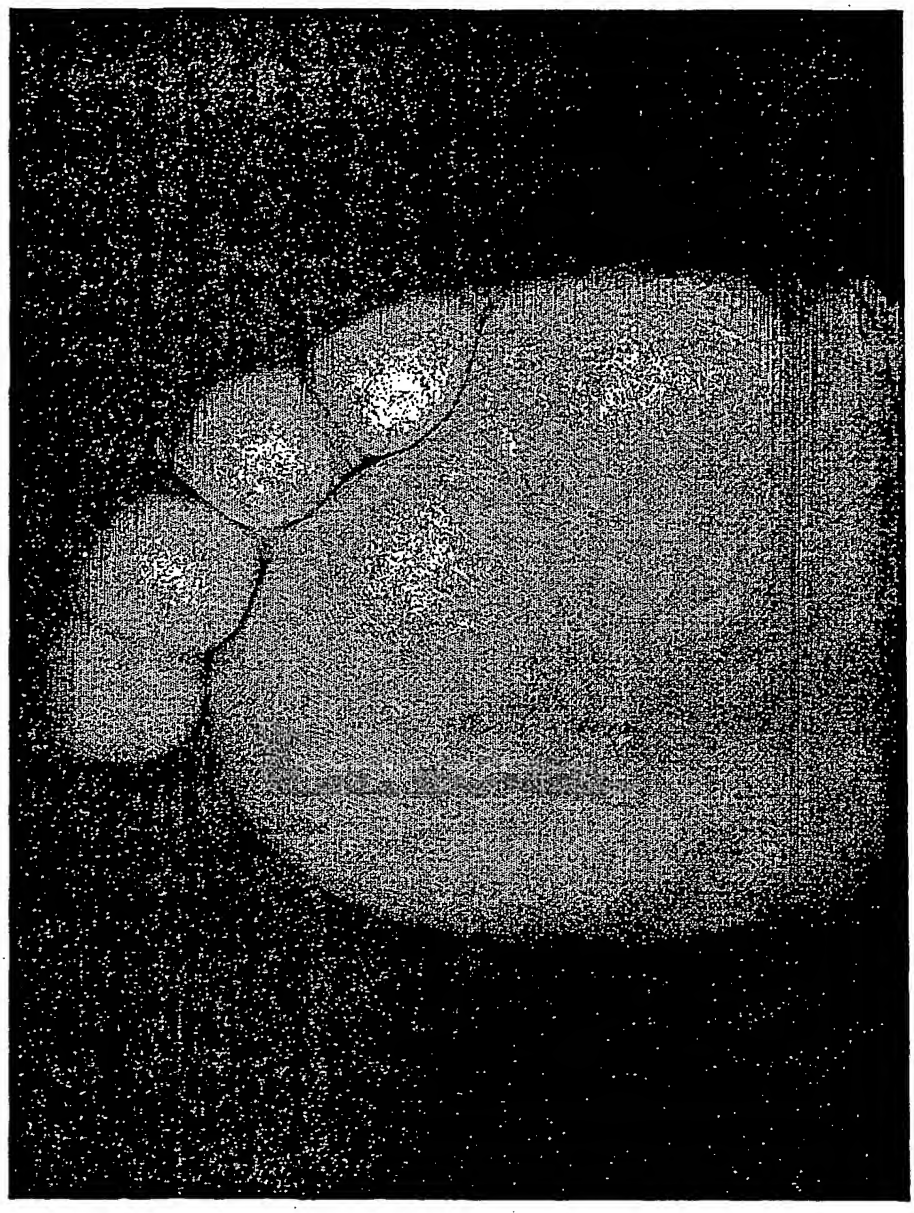


Figure 7i Patient 3: After 1 month follow up

Figure 8

Delivery Vehicle	Wound bed substrate		
	Collagen I	DED-BM	DEM+BM
Cells in suspension	+	+	+
Cultured epithelial autografts	-	-	+
Cells on collagen I coated dressing	-	-	+
Cells on TranCell dressing	-	-	+

INTERNATIONAL SEARCH REPORT

Application No
PCT/GB 02/04829

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/00 A61L15/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 78928 A (HADDOW DAVID ;MACNEIL SHEILA (GB); SHORT ROBERT (GB); UNIV SHEFFIE) 28 December 2000 (2000-12-28) cited in the application	1-7, 10-14, 22-26, 28-32, 36-47,49
Y	column 25, line 24 - line 27; claims; figures	1-49
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 February 2003	24/02/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Winger, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRANCE R M ET AL: "Attachment of human keratinocytes to plasma co-polymers of acrylic acid/octa-1,7-diene and allyl amine/octa-1,7,-diene" JOURNAL OF MATERIALS CHEMISTRY, THE ROYAL SOCIETY OF CHEMISTRY, CAMBRIDGE, GB, vol. 8, no. 1, 1998, pages 37-42, XP002158573 ISSN: 0959-9428 cited in the application	1-7, 9-12, 22-26, 28-32, 38-45
Y	page 38, paragraph 1 p42, conclusion	1-49
X	FRANCE R M ET AL: "Plasma Copolymerization of Allyl Alcohol/1,7-Octadiene: Surface Characterization and Attachment of Human Keratinocytes" CHEM. MATER., vol. 10, 1998, pages 1176-1183, XP002230448 cited in the application	1,8,26, 29-32, 38,42
Y	page 1177, column 1 -page 1178, column 1; figure 7	1-49
X	US 6 101 973 A (EBERT MICHAEL J ET AL) 15 August 2000 (2000-08-15) column 1, line 10 - line 15 column 8, line 15 - line 25	1,6,8,9, 13-17, 38,42
X	WO 89 11500 A (COMMW SCIENT IND RES ORG) 30 November 1989 (1989-11-30) page 3, line 10 -page 6, line 11	1,8,9, 13-15, 22,26, 38,42
X	WO 97 21497 A (CHABRECEK PETER ;CIBA GEIGY AG (CH); LOHMANN DIETER (CH); HOEPKEN) 19 June 1997 (1997-06-19) example B8	1-7, 38-43
X	US 4 836 884 A (MCAUSLAN BRIAN R) 6 June 1989 (1989-06-06) claim 1	1-7
X	LATKANY R ET AL: "Plasma surface modification of artificial corneas for optimal epithelialization" JOURNAL OF BIOMEDICAL MATERIALS RESEARCH, WILEY, NEW YORK, NY, US, vol. 36, no. 1, July 1997 (1997-07), pages 29-37, XP002224793 ISSN: 0021-9304 page 30, column 1	1,8

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/04829

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	O'TOOLE L ET AL: "AN INVESTIGATION OF THE MECHANISMS OF PLASMA POLYMERISATION OF ALLYL ALCOHOL" JOURNAL OF THE CHEMICAL SOCIETY. FARADAY TRANSACTIONS, ROYAL SOCIETY OF CHEMISTRY, CAMBRIDGE, GB, vol. 93, no. 6, 21 March 1997 (1997-03-21), pages 1141-1145, XP000681906 ISSN: 0956-5000 page 1141, column 2	1,8

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter[illegible]l application No.
[illegible]/GB 02/04829

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 36 and 37 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Application No
PCT/GB 02/04829

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0078928 A	28-12-2000	AU 5546400 A	09-01-2001
		CN 1357039 T	03-07-2002
		EP 1185621 A2	13-03-2002
		WO 0078928 A2	28-12-2000
		NO 20016184 A	21-02-2002
US 6101973 A	15-08-2000	US 6049736 A	11-04-2000
		DE 19839996 A1	04-03-1999
		FR 2767837 A1	05-03-1999
		US 6053171 A	25-04-2000
WO 8911500 A	30-11-1989	WO 8911500 A1	30-11-1989
WO 9721497 A	19-06-1997	EP 0834352 A1	08-04-1998
		US 5760100 A	02-06-1998
		AU 709598 B2	02-09-1999
		AU 1139997 A	03-07-1997
		CA 2238345 A1	19-06-1997
		WO 9721497 A1	19-06-1997
		EP 0865326 A1	23-09-1998
		JP 2000503044 T	14-03-2000
		US 6169127 B1	02-01-2001
		AT 188041 T	15-01-2000
		AT 205606 T	15-09-2001
		AT 184708 T	15-10-1999
		AU 703193 B2	18-03-1999
		AU 5147596 A	23-10-1996
		AU 704749 B2	06-05-1999
		AU 5147896 A	23-10-1996
		AU 5814496 A	29-11-1996
		AU 7637396 A	03-07-1997
		BR 9604817 A	09-06-1998
		BR 9604842 A	16-06-1998
		BR 9608824 A	15-06-1999
		CA 2213357 A1	10-10-1996
		CA 2215118 A1	10-10-1996
		CA 2220217 A1	21-11-1996
		CA 2238650 A1	19-06-1997
		CN 1186550 A	01-07-1998
		CZ 9703108 A3	18-03-1998
		CZ 9703122 A3	18-03-1998
		CZ 9703639 A3	18-03-1998
		DE 29624309 U1	03-01-2002
		DE 69604285 D1	21-10-1999
		DE 69604285 T2	23-03-2000
		DE 69605780 D1	27-01-2000
		DE 69605780 T2	20-07-2000
		DE 69615168 D1	18-10-2001
		DE 69615168 T2	16-05-2002
		DK 820601 T3	22-05-2000
		DK 819258 T3	21-01-2002
		EA 1397 B1	26-02-2001
		EE 9700236 A	15-04-1998
		WO 9631791 A1	10-10-1996
		WO 9631792 A1	10-10-1996
		WO 9636890 A1	21-11-1996
		EP 1043605 A1	11-10-2000
		EP 0820601 A1	28-01-1998

INTE[redacted]TIONAL SEARCH REPORT

Int[redacted]ation on patent family members

Int[redacted]plication No
PCT/GB 02/04829

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9721497 A		EP 0819258 A1	21-01-1998
		EP 0826158 A1	04-03-1998
		EP 0865615 A1	23-09-1998
		ES 2142574 T3	16-04-2000
		ES 2166882 T3	01-05-2002
US 4836884 A	06-06-1989	AU 7080387 A	09-09-1987
		WO 8705038 A1	27-08-1987
		EP 0258317 A1	09-03-1988
		JP 63502405 T	14-09-1988